REMARKS/ARGUMENTS

I. Amendments

Claims 1-4, 7-9, 19-24 and 36-39 are pending in the application. All of the pending claims are currently rejected by the Examiner under 35 U.S.C. 112, first paragraph, as not enabled. In this Amendment, Applicants have canceled claims 2-4 and 7-9. Claim 1 has been amended to incorporate the limitations recited in canceled claims 2-4, 7 and 8 (which were dependent on claim 1), and also to recite a step of administering the vector to a cancer cell (*in vivo* or *ex vivo*) by directly injecting the recombinant vectors into a tumor comprising the cancer cell. Support for the amendments to claim 1 can be found, *e.g.*, in the claims as originally filed and in the specification at page 6, lines 1-9; page 20, line 21 to page 21, line 11; and page 22, line 29 to page 23, line 1. Independent claim 19 has been amended to recite a substantial utility for the claimed vector composition: expression of an interferon-α polypeptide in a mammalian cell. Support for this amendment can be found in the claims as originally filed. Claim 34 has been amended to recite methods for inhibiting (not necessarily killing) the growth of hepatocellular carcinoma cells in which interferon-α lacking a secretion leader sequence is expressed, and also to recite a step of administering the vector to a cancer cell (*in vivo* or *ex vivo*) by directly injecting the recombinant vectors into a tumor comprising the cancer cell. Support for the amendments to claim 34 can be found, *e.g.*, in the claims as originally filed and in the specification at page 6, lines 1-9; page 20, line 21 to page 21, line 11; and page 22, line 29 to page 23, line 1.

Applicants have amended their claims without prejudice and expressly reserve the right to pursue claims of equal or greater scope in this application or in a related application. No new matter has been added to any of the pending claims. For the reasons set forth herein, Applicants believe that the each of the Examiner's enablement rejections is overcome.[1]

[1] Applicants and Examiner discussed several of the issues discussed herein during a brief telephonic Interview on August 25, 2004. Although agreement was not reached with respect to the allowability of the previously pending claims, Applicants greatly appreciate the thoughtful comments provided by the Examiner and the Examiner's generous donation of her time.

II. Enablement of Pending Composition Claims 19-33 Under 35 U.S.C. 112[2]

A patentable composition needs only one enabled use. *See* 35 U.S.C. 101; *see also Raytheon v. Roper*, 724 F.2d 951 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 (1984). The MPEP explains that this use must be specific, substantial and credible. *See* MPEP 2164.07. Here, Applicants' claimed compositions have a specific, substantial and credible use: they are useful for expressing interferon-α polypeptides lacking secretion leader sequences in mammalian cells. This utility is recited explicitly in Applicants' amended claims.

Applicants' recited utility is specific, substantial and credible because Applicants have identified a "particular biological activity" for the non-secreted interferon-α polypeptides encoded by their vector and explained how that activity can be utilized by those of skill in the art. *See* MPEP 2107.02.II.A. The anti-proliferative and antiviral properties of non-secreted interferon-α are demonstrated in Figures 4 and 5 of the specification (*see* specification at page 4, lines 19-28). Moreover, the experimental results depicted in Figures 5-8 show that non-secreted interferon-α polypeptides exhibit the biological activity of their secreted counterparts and are equally capable of inducing biologically relevant activities including the phosphorylation of STAT1 and hypophosphorylation of Rb.

The biologically active non-secreted interferon-α proteins encoded by Applicants' claimed vectors can be expressed "in a variety of recombinantly engineered cells," as disclosed by Applicants on page 16, lines 12-15 of the specification. Based on Applicants' disclosure, the specified utility of a vector capable of expressing biologically active non-secreted interferon-α polypeptides is clearly credible, *i.e.*, one of ordinary skill in the art would believe in such a utility. *See* MPEP 2107.02.III.B.

Applicants also note that, in addition to the explicitly provided utility, other specific, substantial and credible utilities of a vector capable of expressing a biologically active interferon-α polypeptide need not be explicitly disclosed when the utility is well-established. *See* MPEP 2107.02.II.B, citing *In re Folkers*, 344 F.2d 970 (CCPA 1965). Section 2107.02.II.B of

[2] Because of the different issues presented, Applicants will deal with the enablement of the pending composition claims separately from the pending method claims.

the MPEP provides "the cloning and characterization of the nucleotide sequence of a well-known protein such as insulin" as an example of a composition with a well-established utility. Similarly, Applicants have not only cloned and characterized vectors encoding a non-secreted interferon-α polypeptide, but they have demonstrated (as discussed above) that the biological activity of the expressed protein is identical for practical purposes to that of secreted interferon-α polypeptides. As of the priority date of Applicants' application, the utility of purified interferon-α polypeptides which were biologically active in mammalian systems was well-recognized. For instance, it was well-known that interferon-α polypeptide could be administered therapeutically to patients with myeloproliferative disorders *(see, e.g.*, the studies cited in the "Background" section of Applicant's specification at page 1, line 17 to page 2, line 5). Thus, the utility of a vector capable of *expressing* an interferon-α peptide in a mammalian cell need not be explicitly disclosed for the utility of such a vector to be recognized by one of ordinary skill in the art. A non-secreted interferon-α polypeptide expressed from Applicants' claimed vectors in mammalian cells is very likely to be folded and processed correctly for maximum activity and, based on Applicants' disclosure, this advantage would be recognized by one skilled in the art. Non-secreted interferon-α polypeptide expressed from Applicants' claimed vectors in mammalian cells could be purified according to any of several methods well-known to those of ordinary skill.[3] As stated above, only *one* specific, substantial and credible utility for a composition needs to be enabled for patentability purposes. Applicants have discovered novel vectors which are useful for expressing biologically active and non-secreted interferon-α polypeptides. Whether Applicants' have taught one of skill in the art how to successfully cure cancer with their vectors is therefore *irrelevant* to the discussion of whether their compositions are, in fact, patentably enabled (*see, e.g.*, MPEP 2107.02.I ("[I]f Applicant makes one credible assertion of utility, utility for the claimed invention as a whole is established"), citing *In re Gottlieb*, 328 F.2d 1016 (CCPA 1964) ("Having found that the antibiotic is useful for some

[3] Applicants submit that enablement of this utility does not require extraordinary or even commercial levels of the protein to be expressed in the cells. Enough protein is expressed that a biochemist of ordinary skill can measure the biological activity of the extracts and purify it to levels that are appropriate for whatever use is envisioned by the biochemist.

purpose, it becomes unnecessary to decide whether it is in fact useful for the other purposes 'indicated' in the specification ...")).

Based on what was known in the art at the time of filing, and in light of what is taught in their specification, Applicants have provided more than sufficient teaching for one of ordinary skill to make and use their recombinant vectors for the expression of non-secreted interferon-α polypeptides which are biologically active in mammalian cells.[4] Applicants submit that the enablement of vectors with the recited utility is supported by the results in the Figures described above and additional experiments *in vivo*. Applicants have taught how to use and test the vectors in the specification at, *e.g.*, Examples 4-8. Moreover, Applicants have demonstrated that their constructs, when injected into hepatocellular tumors in mice, express non-secreted interferon-α which causes those tumors to shrink dramatically *(see* specification at page 20, line 21 to page 21, line 11; Figure 10). Applicants submit that one of ordinary skill would recognize that the methods for making and using the claimed vectors are taught and the biological activity of the non-secreted interferon-α expressed is clearly established. For all the forgoing reasons, the Examiner should withdraw the rejection of Applicants' composition claims under 35 U.S.C. § 112, first paragraph.

Arguments relevant to the enablement of other uses of the vector are discussed below.

III. Enablement of Pending Method Claims 1 and 34-39

The Examiner rejected all of Applicants' pending method claims as being non-enabled. Applicants have amended the previously pending claims. As amended, the claims describe embodiments of Applicants' invention that have not previously been subjected to the Examiner's analysis under 35 U.S.C. 112, first paragraph. Specifically, the amended claims recite methods for expressing interferon-α polypeptides using techniques of direct injection which, together with the use of tissue-specific promoters, greatly diminishes any alleged

[4] Or, to paraphrase the Examiner, Applicants have shown the skilled artisan "how to make the necessary starting materials and ... how to use them to produce the biological effects *as recited in the claims*." *See* January 29, 2004 Office Action at page 3 *(emphasis added)*.

unpredictability and/or safety issues associated with the use of the methods in "gene therapy" applications, *e.g.*, allergic reactions, inappropriate expression, etc. Applicants also note that the claims do not require the "curing" or "destroying" or "killing" of any and all cancerous cells in an animal which might be treated with their claimed method. In addition, the claimed methods may be practiced in conjunction with other methods for effecting anti-proliferation of cells, including those methods described in the specification and other methods known to those of ordinary skill in the art. *See, e.g.*, page 22, lines 21-25 (describing immunosuppressive agents which may be administered with the vectors recited in the claimed methods); page 23, line 23 to page 24, line 12 (describing delivery enhancing agents); and page 25, lines 21-27 (describing *ex vivo* administration to cells or tissues).

The Examiner has expressed doubt that studies showing the successful use of recombinant vectors to treat tumors in xenograft animal models is sufficiently predictive to meet the enablement standard. Applicants disclosed the results of such a study in their specification (*e.g.*, Figure 10). Applicants respectfully submit that the use of xenograft mice (*e.g.*, BALBC nuce mice) to determine whether a test compound is likely to be effective in other animals (*e.g.*, cats, dogs, livestock and humans) is widespread and has been so for years, dating back to at least Applicants' priority date.

With respect to the level of skill required to practice Applicants' claims, Applicants' do not understand the distinction that the Examiner appears to be drawing between artisans with an exceptionally high level of expertise and artisans with exceptional skill. As of Applicants' filing date, there were many skilled practitioners capable of following protocols and performing the routine experimentation -- manipulation of virus titers, identification of tumor cells likely to be targetable, *etc.* -- required to successfully practice Applicants' claimed methods. For example, as early as 1994, effective peritumoral delivery of adenoviruses encoding p53 was demonstrated by Wills et al. (*Human Gene Therapy, 5*:1079, 1081 (1994)) (copy enclosed). Those skilled in the art were aware of this work and their ability to practice the necessary protocols is presumed.

In an earlier Office Action (paper 5), the Examiner cited Gura's 1997 article and quoted Alan Oliff stating that "[animal] model systems are not predictive at all." Applicants note

that in spite of this titillating soundbite, Dr. Oliff continued to regularly direct and publish animal experiments evaluating cancer treatments using xenograft models (a list of seven such articles is attached). Applicants respectfully submit that Dr. Oliff does not continue to use xenograft animal models merely to prove that such models are worthless to scientists. Moreover, the FDA appears to disagree strongly with Dr. Oliff, as the FDA relies heavily on data obtained from such models when evaluating the efficacy and safety of proposed therapeutics.

Throughout the Office Actions, the Examiner has expressed concern that studies using immunocompromised mice such as those described in Applicants' specification (*e.g.*, Figure 10) do not reflect the results that would be observed with immunocompetent mice. Applicants recognize that immunocompromised mice and immunocompetent mice differ in important ways, but respectfully submit that the non-secreted interferon-α polypeptides encoded by Applicants' vector would not be expected to be any more immunogenic than the well-studied secreted form of the protein. The Examiner's position appears to be that a preexisting or induced immune response to an adenoviral vector would render the vector useless upon systemic application. As an initial matter, Applicants' submit that this is essentially an issue of bioavailability which is commonly dealt with in the pharmaceutical field by dosage regimen. So long as the dose sufficient to provide a response is lower than the toxic dose, the agent can exert its therapeutic effect, however small. Applicants' have provided in their previous communications a wealth of evidence that systemic application of adenoviruses does not prevent efficacy. That evidence points to a clear conclusion: the fact that an agent may induce an immune response does not preclude its efficacy.

Applicants have also attached an unpublished but submitted paper by Tsai *et al.* (copy attached) which shows that the replication-deficient (protein IV-deleted) adenovirus vectors recited in Applicants' achieved anti-tumor efficacy in the presence of a pre-existing blocking titer of human anti-adenovirus antibodies. *See, e.g.*, page 4, first full paragraph; pages 4, "Adenovirus vectors"; and page 9, "Effect of human neutralizing antibodies on adenovirus vector function ...." Thus, Applicants submit that their studies using a nude mouse model are predictive and do not differ significantly from results which would be obtained in an "immunocompetent" mouse. This work was specifically designed to address what appears to be

the Examiner's concern: the potential neutralization of systemically administered adenoviruses by a pre-existing immune response. As shown by the data provided in this work, the presence of pre-existing or induced anti-adenoviral antibodies does not preclude the ability of systemically administered adenovirus to cause an antitumor effect in these animal models.

Additionally, the Examiner may question the mouse xenograft model for the evaluation of human adenovirus vectors as predictive of the effects of such vectors when administered to human beings since the human adenovirus vectors preferentially infect human cells leaving much of the mouse privileged from infection by the adenoviral agent. Such an argument is plausible – except for the fact that the human experience in clinical trials with a wide variety of adenoviral vectors has demonstrated a correlation between the effects demonstrated in the preclinical models and the clinical experience and a lack of toxicity associated with these agents. Such an argument could be advanced with respect to any therapeutic compound. If preclinical models were always predictive of the human condition, no compounds would ever fail in the clinic. No animal model is perfectly predictive of the response in the human being but there is sufficient correlation of such models to the human condition for one of skill in the art to believe that agent which demonstrate activity in such models will likely have activity in human beings. The courts have repeatedly held and the USPTO has expressly adopted the policy that human clinical data is not required for enablement of compositions or methods which may be useful for treating human diseases.

In Paper 5, the Examiner also cited the Marshall article for the proposition that "the central challenge in the field of gene therapy is to find safe vectors capable of transporting genes efficiently into target cells and getting the cells to express the genes once they are inserted." *See* Paper 5, page 6. Applicants remind the Examiner that Applicants' pending claims are not drawn to encompass the entire field of adenovirus based gene therapy and Applicants do not pretend to have enabled a gene-based cure for every human disease.

The Marshall article chronicles the discovery of leukemia in children receiving retroviral gene therapy for X-linked severe combined immunodeficiency (X-SCID). Applicants submit that the Marshall article does not show that those skilled in the art of developing recombinant adenovirus treatments for cancer cannot reasonably predict whether a particular

construct will have any functionality or effectiveness in a clinical setting. Rather, the articles demonstrate only that treating certain particularly severe *genetic diseases* with the retrovirus-based or adenovirus-based protocols described in the articles is accompanied by serious risks. Applicants remind the Examiner that *nearly all* existing methods of treating tumors with pharmaceuticals are accompanied by the risk of serious, often life-shortening, side-effects. To the extent that the absence of any risk is neither explicitly nor implicitly included in the pending claims, the Examiner's implicit reliance on such risks to reject the pending claims is misplaced and contrary to law. *See, e.g., Scott v. Finney*, 34 F.3d 1058, 1063 (Fed. Cir. 1994) ("Testing for full safety and effectiveness . . . is more properly left to the [FDA].").[5]

Applicants also respectfully draw the Examiner's attention to an apparent double-standard in the Patent Office's approach to these issues. In 1997, David T. Curiel filed an application for a U.S. patent with claims reciting methods for transducing cells with replication-deficient adenoviral vectors. The purpose of the methods as stated in the specification of Dr. Curiel's patent is to allow "effective genetic correction in the context of gene therapy." The patent (U.S. Patent No. 6,333,030) issued Christmas Day, 2001. The Examples provided in Dr. Curiel's patent specification describe the transduction of a xenografted tumor in mice and Applicants' pending claims appear to be of similar scope to those issued to Dr. Curiel. Dr. Curiel did *not* provide any clinical data in the patent specification or by declaration during prosecution. Thus, Dr. Curiel's implicit opinion that, at least as early as 1997, xenografted tumors were useful for predicting adenovirus-based treatments in humans was shared by the Patent Office at least as recently as Christmas Day, 2001. Applicants recognize that the decisions of the Patent Office with respect to the allowance of another's claims are not binding on all parties. Nevertheless, Applicants remain puzzled by the seemingly contradictory approach taken by the Patent Office with respect to the efficacy, predictability and enablement of "gene therapy" claims.

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[5] Applicants also wish to point out that the FDA's requirements for "safety" and predictability are often higher than that deemed acceptable to scientists and medical practitioners seeking to treat terminally ill people who have no other alternatives. *See, e.g.,* Bailey, R., *ReasonOnline*, "Timid Bureaucrats Kill People," (Jan. 9, 2002) (copy attached).

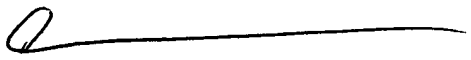
In summary, Applicants' position is that, in light of their teaching and the knowledge of those skilled in the art, the use of Applicants' *xenograft-proven* recombinant adenovirus is *reasonably likely* to (1) successfully express the encoded interferon-α polypeptide and (2) effectively diminish the growth of at least *some* transfected tumor cells in the targeted population, without undue experimentation. Applicants' respectfully submit that to satisfy the enablement requirement they need not prove that the *risk-free elimination* of tumors occurs inevitably when their claimed methods are practiced.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,



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Mary Ann Liebert, Inc., Publishers

Development and Characterization of Recombinant Adenoviruses Encoding Human p53 for Gene Therapy of Cancer

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ABSTRACT

We have constructed recombinant human adenoviruses that express wild-type human p53 under the control of either the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) immediate early gene promoter. Each construct replaces the Ad 5 E1a and E1b coding sequences necessary for viral replication with the p53 cDNA and MLP or CMV promoter. These p53/Ad recombinants are able to express p53 protein in a dose-dependent manner in infected human cancer cells. Tumor suppressor activity of the expressed p53 protein was assayed by several methods. [^{3}H]Thymidine incorporation assays showed that the recombinant adenoviruses were capable of inhibiting DNA synthesis in a p53-specific, dose-dependent fashion. *Ex vivo* treatment of Saos-2 tumor cells, followed by injection of the treated cells into nude mice, led to complete tumor suppression using the MLP/p53 recombinant. Following a single injection of CMV/p53 recombinant adenovirus into the peritumoral space surrounding an *in vivo* established tumor derived from a human small cell lung carcinoma cell line (NIH-H69), we were able to detect p53 mRNA in the tumors at 2 and 7 days post-injection. Continued treatment of established H69 tumors with MLP/p53 recombinant led to reduced tumor growth and increased survival time compared to control treated animals. These results indicate that recombinant adenoviruses expressing wild-type p53 may be useful vectors for gene therapy of human cancer.

OVERVIEW SUMMARY

Introduction of the p53 tumor suppressor gene into tumors bearing p53 mutations can inhibit cellular proliferation and tumorigenicity. Wills *et al.* describe replication-deficient recombinant adenoviruses directing expression of human p53 both *in vitro* and *in vivo*. They show that adenovirus-mediated expression of wild-type p53 in p53 altered tumors can suppress proliferation and inhibit tumorigenicity in *ex vivo* and *in vivo* cancer models.

INTRODUCTION

MUTATION OF THE P53 GENE is the most common genetic alteration in human cancers (Bartek *et al.*, 1991; Hollstein *et al.*, 1991). In its proposed role as a "guardian of the genome" (Lane, 1992), the p53 gene product functions as a transcriptional activator of other genes which inhibit cell cycle progression from G_1 to S phase in normal cells. Its levels rise and accumulate in response to DNA damage, leading either to G_1 arrest and repair, terminal differentiation, or, if too much damage has occurred, apoptosis (Kuerbitz *et al.*, 1992; Lane, 1992). Loss of wild-type p53 function is associated with the uncontrolled growth of many types of human cancers. The reexpression of normal p53 in p53-altered tumor cells has been demonstrated to suppress tumor growth (Chen *et al.*, 1990; Cheng *et al.* 1992; Takahashi *et al.*, 1992) or induce apoptosis (Yonish-Rouach *et al.*, 1991, Shaw *et al.*, 1992). Therefore, p53 functions as a tumor suppressor, restoring a nontumorigenic phenotype to tumor cells in which the endogenous p53 gene has been deleted or mutated.

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Recent work has shown that human adenoviruses can be used to deliver genes successfully into a variety of cells and tissues (Lemarchand *et al.*, 1992; Rosenfeld *et al.*, 1992; Rich *et al.*, 1993). Recombinant adenoviruses have several advantages over alternative gene delivery systems such as retrovirus (RV) or adeno-associated virus (AAV)-based vectors for the treatment of cancer. These include the ability to produce stable, high-titer virus capable of efficient infection and subsequent gene expression in target cells (for review, see Siegfried, 1993). Because of the advantages of an adenovirus-based delivery system over other systems for the potential gene therapy of cancer, we constructed recombinant adenoviruses encoding wild-type p53 under the control of the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) promoter. We have tested the ability of these constructs to suppress tumor growth both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines

Recombinant adenoviruses were grown and propagated in the human embryonal kidney cell line 293 (ATCC CRL 1573) maintained in DME medium containing 10% defined, supplemented calf serum (Hyclone). Saos-2 cells were maintained in Kaighn's media supplemented with 15% fetal calf serum. HeLa and Hep 3B cells were maintained in DME medium supplemented with 10% fetal calf serum. All other cell lines were grown in Kaighn's media supplemented with 10% fetal calf serum. Saos-2 cells were kindly provided by Dr. Eric Stanbridge. All other cell lines were obtained from ATCC.

Construction of recombinant adenoviruses

To construct the Ad5/p53 viruses, a 1.4-kb *Hind* III–*Sma* I fragment containing the full-length cDNA for p53 was isolated from pGEM1-p53-B-T (kindly supplied by Dr. Wen-Hwa Lee) and inserted into the multiple cloning site of the expression vector pSP72 (Promega) using standard cloning procedures (Sambrook *et al.*, 1989). The p53 insert was recovered from this vector following digestion with *Xho* I–*Bgl* II and gel electrophoresis. The p53 coding sequence was then inserted into either pNL3C or pNL3CMV adenovirus gene transfer vectors (kindly provided by Dr. Robert Schneider), which contain the Ad5 5′ inverted terminal repeat and viral packaging signals and the E1a enhancer upstream of either the Ad2 major late promoter (MLP) or the human cytomegalovirus immediate early gene promoter (CMV), followed by the tripartite leader cDNA and Ad 5 sequence 3,325–5,525 bp in a pML2 background. These new constructs replace the E1 region (bp 360–3,325) of Ad5 with p53 driven by either the Ad2 MLP (A/M/53) or the human CMV promoter (A/C/53), both followed by the tripartite leader cDNA (see Fig. 1). The p53 inserts use the remaining downstream E1b polyadenylation site. Additional MLP- and CMV-driven p53 recombinants (A/M/N/53, A/C/N/53) were generated which had a further 705-nucleotide deletion of Ad 5 sequence to remove the protein IX (pIX) coding region. As a control, a recombinant adenovirus was generated from the parental pNL3C plasmid without a p53 insert (A/M). A second

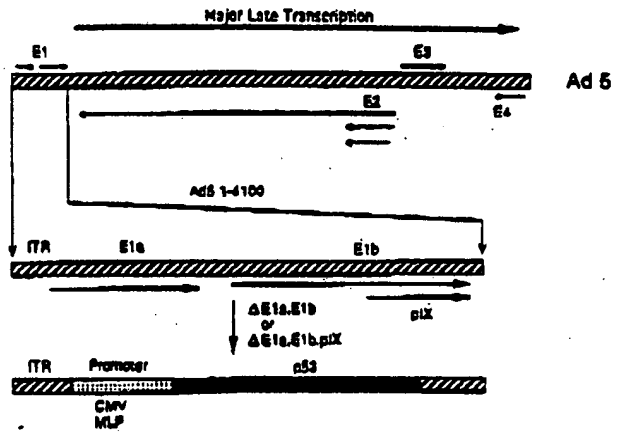

FIG. 1. Schematic of recombinant p53/adenovirus constructs. The p53 recombinants are based on Ad 5 and have had the E1 region of nucleotides 360–3,325 replaced with a 1.4-kb full-length p53 cDNA driven by the Ad 2 MLP (A/M/53) or human CMV (A/C/53) promoters followed by the Ad 2 tripartite leader cDNA. The control virus A/M has the same Ad 5 deletions as the A/M/53 virus, but lacks the 1.4-kb p53 cDNA insert. The remaining E1b sequence (705 nucleotides) have been deleted to create the protein IX-deleted constructs A/M/N/53 and A/C/N/53. These constructs also have a 1.9-kb *Xba* I deletion within adenovirus type 5 region E3.

control (kindly provided by Dr. Robert Schneider) consisted of a recombinant adenovirus encoding the β-galactosidase (β-Gal) gene under the control of the CMV promoter (A/C/β-Gal). The plasmids were linearized with either *Nru* I or *Eco* RI and co-transfected with the large fragment of a *Cla* I-digested Ad 5 *dl*309 or *dl*327 mutants (Jones and Shenk, 1979; Thimmappaya *et al.*, 1982) using a Ca/PO_4 transfection kit (Stratagene). Only the pIX-minus constructs used the *dl*327 background which contains a 1.9-kb *Xba* I deletion in the E3 region. Viral plaques were isolated and recombinants identified by both restriction digest analysis and the polymerase chain reaction (PCR) using recombinant-specific primers against the tripartite leader cDNA sequence with downstream p53 cDNA sequence. Recombinant virus was further purified by limiting dilution, and virus particles were purified and titered by standard methods (Graham and van der Erb, 1973; Graham and Prevec, 1991).

p53 protein detection

Saos-2 or Hep 3B cells (5×10^5) were infected with the indicated recombinant adenoviruses for a period of 24 hr at increasing multiplicities of infection (moi) of plaque-forming units of virus/cell. Purified adenovirus, stored in 1% sucrose in phosphate-buffered saline (PBS), is diluted with media to obtain the desired moi and added to plates of cells containing fresh media. After 24 hr, the cells were washed once with PBS and harvested in lysis buffer [50 m*M* Tris-HCl pH 7.5, 250 m*M* NaCl, 0.1% NP-40, 50 m*M* NaF, 5 m*M* EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]. A Bradford assay (Bio-Rad Protein Assay kit) was used to measure cellular protein concentration, and equal amounts of protein (approximately 30 μg) were separated

by 10% SDS-PAGE and transfered to nitrocellulose. Membranes were incubated with α-p53 antibody PAb 1801 (Novocastro) followed by sheep anti-mouse IgG conjugated with horseradish peroxidase. p53 protein was visualized by chemiluminescence (ECL kit, Amersham) on Kodak XAR-5 film.

Measurement of DNA synthesis rate

Cells (5×10^3/well) were plated in 96-well titer plates (Costar) and allowed to attach overnight (37°C, 7% CO_2). Cells were then infected for 24 hr with purified recombinant virus particles at moi values ranging from 0.3 to 100, as indicated. Media were changed 24 hr after infection, and incubation was continued for a total of 72 hr. [^{3}H]Thymidine (Amersham, 1 μCi/well) was added 18 hr prior to harvest. Cells were harvested on glass fiber filters and levels of incorporated radioactivity were measured in a beta scintillation counter. [^{3}H]Thymidine incorporation was expressed as the mean % (±SD) of media control and plotted versus the moi.

Tumorigenicity in nude mice

Approximately 2.4×10^6 Saos-2 cells, plated in T225 flasks, were treated with suspension buffer (1% sucrose in PBS) containing either A/M/N/53- or A/M-purified virus at an moi of 3 or 30. Following an overnight infection, cells were injected subcutaneously into the left and right flanks of BALB/c athymic nude mice (4 mice per group). One flank was injected with the A/M/N/53-treated cells, while the contralateral flank was injected with the control A/M-treated cells, each mouse serving as its own control. Animals receiving bilateral injection of buffer-treated cells served as additional controls. Tumor dimensions (length, width, and height) and body weights were then measured twice per week over an 8-week period. Tumor volumes were estimated for each animal, assuming a spherical geometry with radius equal to one-half the average of the measured tumor dimensions.

Intratumoral RNA analysis

Female BALB/c athymic nude mice (approximately 5 weeks of age) were injected subcutaneously with 1×10^7 H69 small cell lung carcinoma (SCLC) cells in a 200-μl volume in their right flanks. Tumors were then allowed to progress for 32 days. Mice then received peritumoral injections of either A/C/53 or A/C/β-Gal recombinant adenovirus [2×10^9 plaque-forming units (pfu)] into the subcutaneous space beneath the tumor mass. Tumors were excised from the animals 2 and 7 days post adenovirus treatment and rinsed with PBS. Tumor samples were homogenized, and total RNA was isolated using a TriReagent kit (Molecular Research Center, Inc.). Poly(A) RNA was isolated using the PolyATract mRNA Isolation System (Promega), and approximately 10 ng of sample was used for reverse transcriptase (RT)-PCR determination of recombinant p53 mRNA expression (Wang *et al.*, 1989). Primers were designed to amplify sequence between the adenovirus tripartite leader cDNA and the downstream p53 cDNA, ensuring that only recombinant, and not endogenous p53 would be amplified.

p53 gene therapy of established tumors in nude mice

Approximately 1×10^7 H69 (SCLC) tumor cells in 200-μl volumes were injected subcutaneously into female BALB/c athymic nude mice. Tumors were allowed to develop for 2 weeks, at which point animals were randomized by tumor size ($n = 5$/group). Peritumoral injections of either A/M/N/53 or the control A/M adenovirus (2×10^9 pfu/injection) or buffer alone (1% sucrose in PBS) were administered twice per week for a total of 8 doses/animal per group. Tumor dimensions and body weights were measured twice per week for 7 weeks, and tumor volume was estimated as described previously. Animals were then followed to observe the effect of treatment on mouse survival.

RESULTS

Construction of recombinant p53-adenovirus

p53 adenoviruses were constructed by replacing a portion of the E1a and E1b region of adenovirus type 5 with p53 cDNA under the control of either the Ad2 MLP (A/M/53) or CMV (A/C/53) promoter (schematized in Fig. 1). This E1 substitution severely impairs the ability of the recombinant adenoviruses to replicate, restricting their propagation to 293 cells that supply Ad 5 E1 gene products *in trans* (Graham *et al.*, 1977). After identification of p53 recombinant adenovirus by both restriction digest and PCR analysis, the entire p53 cDNA sequence from one of the recombinant adenoviruses (A/M/53) was sequenced to verify that it was free of mutations. Following this, purified preparations of the p53 recombinants were used to infect HeLa cells to assay for the presence of phenotypically wild-type adenovirus. HeLa cells, which are nonpermissive for replication of E1-deleted adenovirus, were infected with $1–4 \times 10^9$ infectious units of recombinant adenovirus at an moi = 50, cultured for 3 weeks, and observed for the appearance of cytopathic effect (CPE). Using this assay, we were not able to detect recombinant adenovirus replication or wild-type contamination, readily evident by the CPE observed in control cells infected with wild-type adenovirus at a level of sensitivity of approximately 1 in 10^9.

p53 protein expression from recombinant adenovirus

To determine if our p53 recombinant adenoviruses expressed p53 protein, we infected tumor cell lines that do not express endogenous p53 protein. The human tumor cell lines Saos-2 (osteosarcoma) and Hep 3B (hepatocellular carcinoma), which contain mutations that result in no expression of p53 protein (Chen *et al.*, 1990; Hsu *et al.*, 1993), were infected for 24 hr with the p53 recombinant adenoviruses A/M/53 or A/C/53 at moi values ranging from 0.1 to 200 pfu/cell. Western analysis of lysates prepared from infected cells demonstrated a dose-dependent p53 protein expression in both cell types (Fig. 2). Both cell lines expressed higher levels of p53 protein following infection with A/C/53 than with A/M/53 (Fig. 2). No p53 protein was detected in noninfected cells. Cells infected with moi values of up to 200 of the control virus A/M also did not show detectable p53 protein (unpublished observation). SW 480 cell

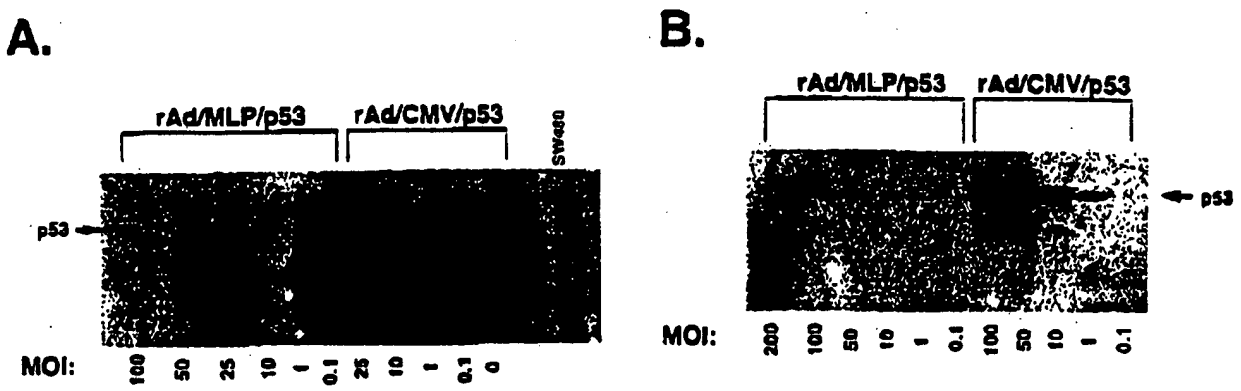

FIG. 2. p53 protein expression in tumor cells infected with A/M/53 and A/C/53. A. Saos-2 (osteosarcoma) cells were infected at the indicated moi with either the A/M/53- or A/C/53-purified virus and harvested 24 hr later. The p53 antibody pAb 1801 was used to stain immunoblots of samples loaded at equal total protein concentrations. Equal protein concentrations of SW480 cell extracts, which overexpress mutant p53 protein, were used as a marker for p53 size. The zero (0) under the A/C/53 heading indicates a mock infection containing untreated Saos-2 lysate. B. Hep 3B (hepatocellular carcinoma) cells were infected with the A/M/53 or A/C/53 virus at the indicated moi and analyzed as in A. The arrow indicates the position of the p53 protein.

lysate, which overexpresses mutant p53 protein (Baker *et al.*, 1990), was used as a size marker. Levels of endogenous wild-type p53 are normally quite low, and nearly undetectable by Western analysis of cell extracts (Bartek *et al.*, 1991). It is clear however that wild-type p53 protein levels are easily detectable after infection with either A/M/53 or A/C/53 at the lower moi values (Fig. 2), suggesting that even low doses of p53 recombinant adenoviruses can produce potentially efficacious levels of p53.

p53-dependent morphology changes

The reintroduction of wild-type p53 into the p53-negative osteosarcoma cell line, Saos-2, results in a characteristic enlargement and flattening of these normally spindle-shaped cells (Chen *et al.*, 1990). Subconfluent Saos-2 cells (1×10^5 cells/10-cm plate) were infected at an moi of 50 with either the A/C/53 or control A/M virus, and incubated at 37°C for 72 hr until uninfected control plates were confluent. At this point, the expected morphological change was evident in the A/C/53-treated plate (Fig. 3C), but not in uninfected (Fig. 3A) or control virus-infected plates (Fig. 3B). This effect was not a function of cell density because a control plate initially seeded at lower density retained normal morphology at 72 hr when its confluence approximated that of the A/C/53-treated plate (data not shown). Our previous results had demonstrated a high level of p53 protein expression at a moi of 50 in Saos-2 cells (Fig. 2A), and these results provided evidence that the p53 protein expressed by these recombinant adenoviruses was biologically active.

p53 inhibition of cellular DNA synthesis

To test further the activity of the p53 recombinant adenoviruses, we assayed their ability to inhibit proliferation of human tumor cells as measured by the uptake of [^{3}H]thymidine. It has previously been shown that introduction of wild-type p53 into cells that do not express endogenous wild-type p53 can arrest the cells at the G_1/S transition, leading to inhibition of uptake of labeled thymidine into newly synthesized DNA (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990). We infected a variety of p53-deficient tumor cell lines with either A/M/N/53, A/C/N/53 or a non-p53-expressing control recombinant adenovirus (A/M). We observed a strong, dose-dependent inhibition of DNA synthesis by both the A/M/N/53 and A/C/N/53 recombinants in 7 out of the 9 different tumor cell lines tested (Fig. 4). Both constructs were able to inhibit DNA synthesis specifically in these human tumor cells, regardless of whether they ex-

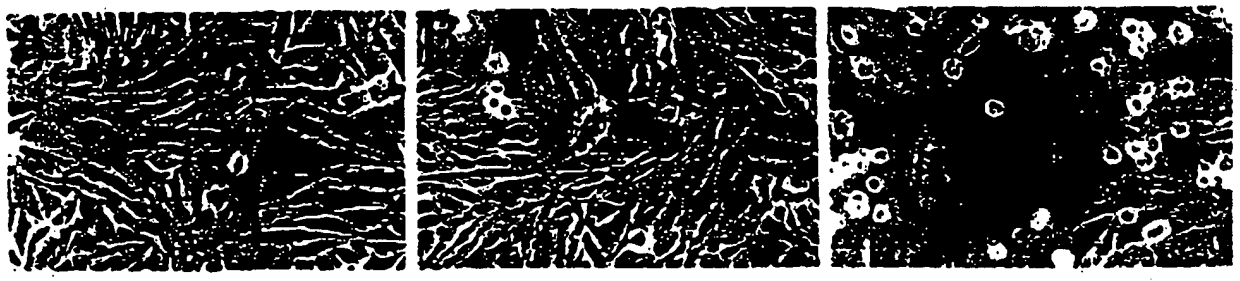

FIG. 3. p53-dependent Saos-2 morphology change. Subconfluent (1×10^5 cells/10-cm plate) Saos-2 cells were either uninfected (A), infected at a moi = 50 with the control A/M virus (B), or the A/C/53 virus (C). The cells were photographed 72 hr post-infection.

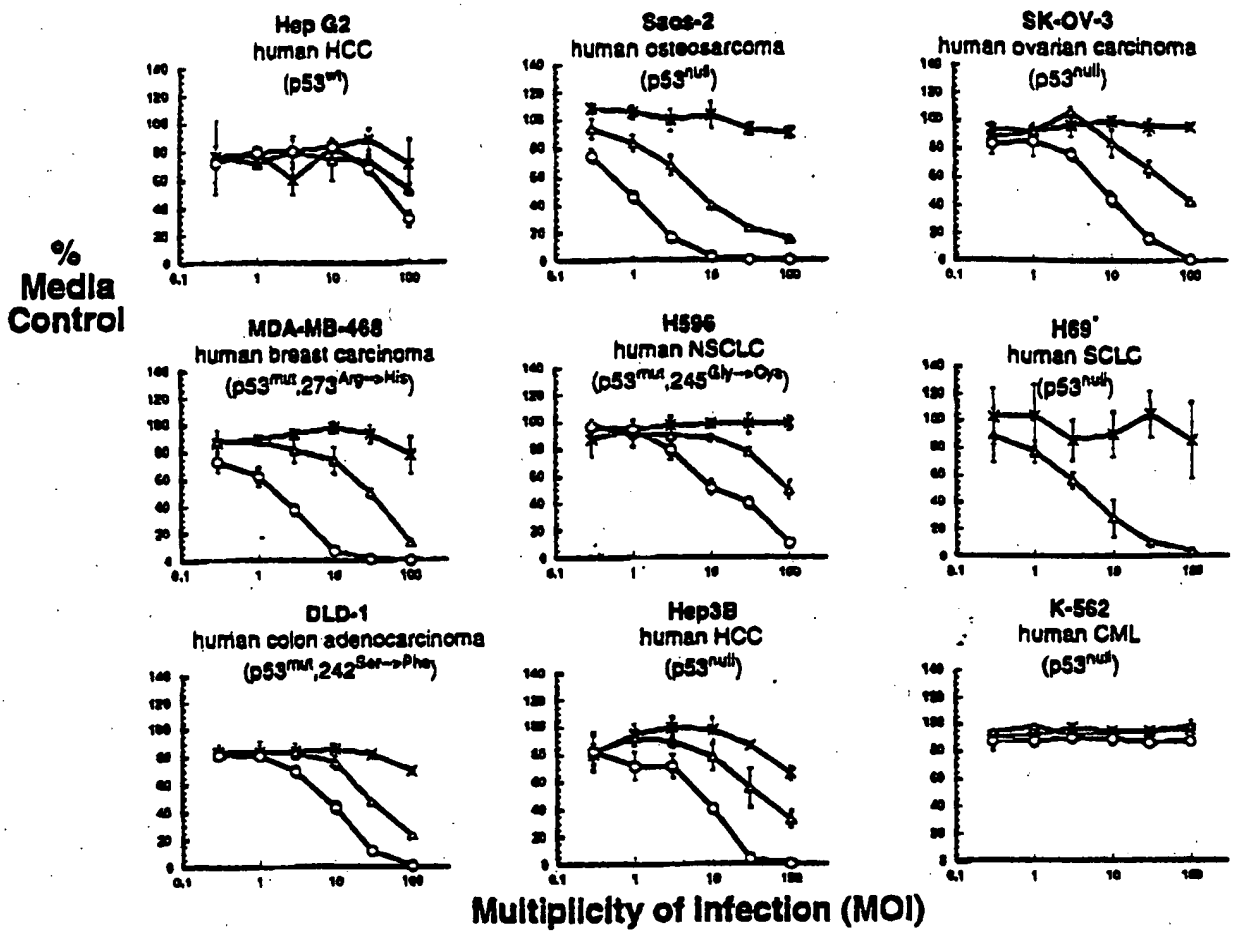

FIG. 4. p53-dependent inhibition of DNA synthesis in human tumor cell lines by A/M/N/53 and A/C/N/53. Nine different tumor cell lines were infected with either control adenovirus A/M/ (xx), or the p53-expressing A/M/N/53 (Δ) or A/C/N/53 (O) virus at increasing moi as indicated. Tumor type and p53 status are noted for each cell line (wt, wild type; null, no protein expressed; mut, mutant protein expressed). DNA synthesis was measured 72 hr post-infection as described in Materials and Methods. Results are from triplicate measurements at each dose (mean ± SD), and are plotted as % of media control *versus* moi. (*) H69 cells were only tested with A/M and A/M/N/53 virus.

pressed mutant p53 or failed to express p53 protein. We also found that in this assay, the A/C/N/53 construct was consistently more potent than the A/M/N/53. In Saos-2 (osteosarcoma) and MDA-MB468 (breast cancer) cells, nearly 100% inhibition of DNA synthesis was achieved with the A/C/N/53 construct at a moi as low as 10. At doses where inhibition by the control adenovirus is only 10–30%, we observed a 50–100% reduction in DNA synthesis using either p53 recombinant adenovirus. In contrast, we observed no significant p53-specific effect with either construct as compared to control virus in HEP G2 cells (hepatocarcinoma cell line expressing endogenous wild-type p53; Bressac *et al.*, 1990), nor in the K562 (p53 null; Feinstein *et al.*, 1992) leukemic cell line.

Tumorigenicity in nude mice

In a more stringent test of function for our p53 recombinant adenoviruses, we infected tumor cells *ex vivo* and then injected the cells into nude mice to assess the ability of the recombinants to suppress tumor growth *in vivo*. Saos-2 cells infected with A/M/N/53 or control A/M virus at a moi of 3 or 30 were injected into opposite flanks of nude mice. Tumor sizes were then measured twice a week over an 8-week period. At a moi of 30, we did not observe any tumor growth in the p53-treated flanks in any of the animals, while the control treated tumors continued to grow (Fig. 5). The progressive enlargement of the control virus-treated tumors was similar to that observed in the buffer-treated control animals. We also observed a clear difference in tumor growth between the control adenovirus and the p53 recombinant at a moi of 3, although tumors from 2 out of the 4 p53-treated mice did start to show some growth after approximately 6 weeks (data not shown). Thus, the A/M/N/53 recombinant adenovirus is able to mediate p53-specific tumor suppression in an *in vivo* environment. We have also observed very similar results when infecting and injecting the NSCLC cell line H596, which expresses mutant p53 protein with the same viruses (unpublished observations).

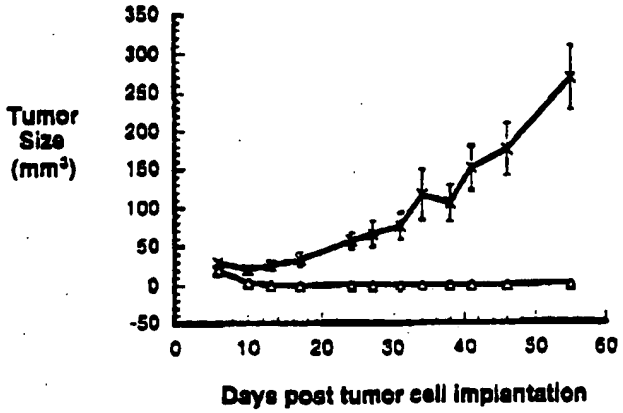

FIG. 5. Tumorigenicity of p53-infected Saos-2 cells in nude mice. Saos-2 cells were infected with either the control A/M virus or the p53 recombinant A/M/N/53 at moi = 30. Treated cells were injected subcutaneously into the flanks of nude mice, and tumor dimensions were measured (as described in Materials and Methods) twice per week for 8 weeks. Results are plotted as tumor size *versus* days post tumor cell implantation for both control A/M-(x) and A/M/N/53-(Δ) treated cells. Error bars represent the mean tumor size ± SEM for each group of 4 animals at each time point.

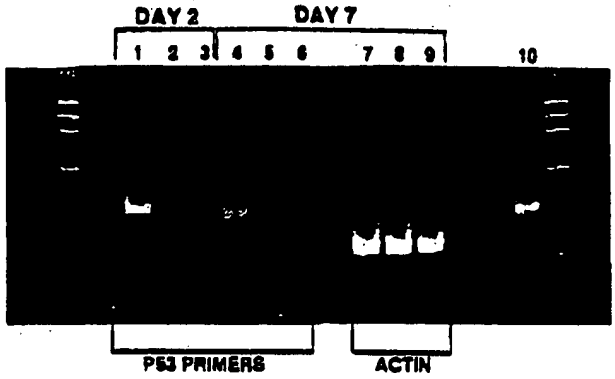

FIG. 6. Expression of rAd/p53 RNA in established tumors. H69 (SCLC) cells were injected subcutaneously into nude mice and allowed to develop tumors for 32 days until reaching a size of approximately 25–50 mm^3. Mice were randomized and injected peritumorally with 2×10^9 pfu of either control A/C/β-Gal or A/C/53 virus. Tumors were excised 2 and 7 days post injection, and poly(A) RNA was prepared from each tumor sample. RT-PCR was carried out using equal RNA concentrations and primers specific for recombinant p53 message. PCR amplification was for 30 cycles at 94°C 1 min, 55°C 1.5 min, 72°C 2 min, and a 10-min, 72°C final extension period in an Omnigen thermalcycler (Hybaid). The PCR primers used were a 5' Tripartite Leader cDNA (5'-CGCCACCGAGGGACCTGAGCGAGTC-3') and a 3' p53 primer (5'-TTCTGGGAAGGGACAGAAGA-3'). Lanes 1, 2, 4, and 5, p53-treated samples excised at days 2 or 7 as indicated; lanes 3 and 6, from β-Gal-treated tumors; lanes 7, 8, and 9, replicates of lanes 4, 5, and 6, respectively, amplified with actin primers to verify equal loading; lane 10, a positive control using a tripartite/p53 containing plasmid.

In vivo *expression of rAd/p53*

Although *ex vivo* treatment of cancer cells and subsequent injection into animals provided a critical test of tumor suppression, a more clinically relevant experiment is to determine if injected p53 recombinant adenovirus could infect and express p53 in established tumors *in vivo*. To address this, H69 (SCLC, $p53^{null}$) cells were injected subcutaneously into nude mice, and tumors were allowed to develop for 32 days. At this time, a single injection of 2×10^9 pfu of either A/C/53 or A/C/β-Gal adenovirus was injected into the peritumoral space surrounding the tumor. Tumors were then excised at either day 2 or day 7 following the adenovirus injection, and poly(A) RNA was isolated from each tumor. RT-PCR, using recombinant-p53 specific primers, was then used to detect p53 mRNA in the p53-treated tumors (Fig. 6, lanes 1, 2, 4, 5). No p53 signal was evident from the tumors excised from the β-Gal-treated animals (Fig. 6, lanes 3 and 6). Amplification with actin primers served as a control for the RT-PCR reaction (Fig. 6, lanes 7–9), while a plasmid containing the recombinant-p53 sequence served as a positive control for the recombinant-p53-specific band (Fig. 6, lane 10). This experiment demonstrates that a p53 recombinant adenovirus can specifically direct expression of p53 mRNA within established tumors following a single injection into the peritumoral space. It also provides evidence for *in vivo* viral persistence for at least 1 week following infection with a p53 recombinant adenovirus.

In vivo efficacy

To address the feasibility of gene therapy of established tumors, a tumor-bearing nude mouse model was used. H69 cells were injected into the subcutaneous space on the right flank of mice, and tumors were allowed to grow for 2 weeks. Mice then received peritumoral injections of buffer or recombinant virus twice weekly for a total of 8 doses. In the mice treated with buffer or control A/M virus, tumors continued to grow rapidly throughout the treatment, whereas those treated with the A/M/N/53 virus grew at a greatly reduced rate (Fig. 7A). Although control animals treated with buffer alone had accelerated tumor growth as compared to either virus-treated group, we found no significant differences in body weight among the three groups during the treatment period (data not shown). Tumor ulceration in some animals limited the relevance of tumor size measurements after day 42. However, continued monitoring of the animals to determine survival time demonstrated a survival advantages for the p53-treated animals (Fig. 7B). The last of the control adenovirus-treated animals died on day 83, while buffer alone treated controls had all expired by day 56. In contrast, all 5 animals treated with the A/M/N/53 survived up to day 137 before the first animal in this group died (Fig. 7B). Two animals continue to survive at day 174. Together, our data indicate a p53-specific effect on both tumor growth and survival time in animals with established p53-deficient tumors.

DISCUSSION

Adenovirus vectors expressing p53

We have constructed recombinant human adenovirus vectors that are capable of expressing high levels of wild-type p53

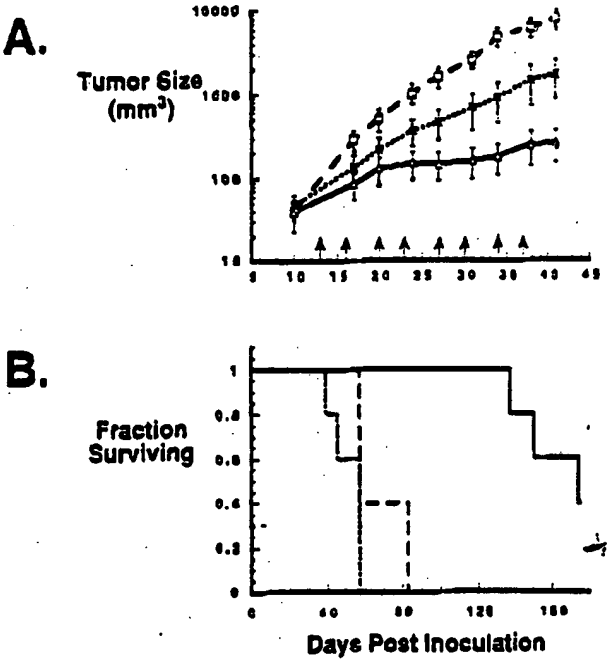

FIG. 7. *In vivo* tumor suppression and increased survival time with A/M/N/53. H69 (SCLC) tumor cells were injected subcutaneously into nude mice and allowed to develop for 2 weeks. Peritumoral injections of either buffer alone (□), control A/M adenovirus (x), or A/M/N/53 (Δ) (both virus 2×10^9 pfu/injection) were administered twice per week for a total of 8 doses. Tumor dimensions were measured twice per week and tumor volume was estimated as described in Materials and Methods. A. Tumor size is plotted for each virus *versus* time (days) post inoculation of H69 cells. Error bars indicate the mean tumor size ± SEM for each group of 5 animals. Arrows indicate days of virus injections. B. Mice were monitored for survival and the fraction of mice surviving per group *versus* time post inoculation of buffer alone (—), control A/M (— —), or A/M/N/53 (——) virus-treated H69 cells is plotted.

protein in a dose-dependent manner. Each vector contains deletions in the E1a and E1b regions that render the virus replication deficient (Challberg and Kelly, 1979; Horowitz, 1991). Of further significance is that these deletions include those sequences encoding the E1b 19- and 55-kD proteins. The 19-kD protein is reported to be involved in inhibiting apoptosis (Rao *et al.*, 1992; White *et al.*, 1992), whereas the 55-kD protein is able to bind wild-type p53 protein (Sarnow *et al.*, 1982; Heuvel *et al.*, 1990). By deleting these adenoviral sequences, we remove potential inhibitors of p53 function through direct binding to p53 or potential inhibition of p53-mediated apoptosis. We have created additional constructs that have had the remaining 3′ E1b sequence, including all protein IX coding sequence, deleted as well. Although this has been reported to reduce the packaging size capacity of adenovirus to approximately 3 kb, less than wild-type virus (Ghosh-Choudhury *et al.*, 1987), these constructs are also deleted in the E3 region so that the A/M/N/53 and A/C/N/53 constructs are well within this size range. By deleting the pIX region, adenoviral sequences homologous to those contained in 293 cells are reduced to approximately 300 bp, decreasing the chances of regenerating replication-competent, wild-type adenovirus through recombination. Constructs lacking pIX coding sequence appear to have equal efficacy and drive equivalent levels of p53 protein expression as those with pIX (unpublished observations).

p53/Adenovirus efficacy in vitro

In concordance with a strong dose dependency for expression of p53 protein in infected cells, we have also demonstrated a dose-dependent, p53-specific inhibition of tumor cell growth by our recombinants. We were able to inhibit cell division, demonstrated by the inhibition of DNA synthesis, in a wide variety of tumor cell types known to lack wild-type p53 protein expression. Baccheti and Graham (1993) recently reported p53-specific inhibition of DNA synthesis in the ovarian carcinoma cell line SKOV-3 by a p53 recombinant adenovirus in similar experiments. In addition to ovarian carcinoma, we have demonstrated that additional human tumor cell lines, representative of clinically important human cancers and including lines overexpressing mutant p53 protein, can also be growth inhibited by our p53 recombinants. At moi values where the A/C/N/53 recombinant is 90–100% effective in inhibiting DNA synthesis in these tumor types, control adenovirus-mediated suppression is less than 20%.

Although Feinstein *et al.* (1992) reported that reintroduction of wild-type p53 could induce differentiation and increase the proportion of cells in G_1 *versus* $S + G_2$ for leukemic K562 cells, we found no p53-specific effect in this line. Horvath and Weber (1988) have reported that human peripheral blood lymphocytes are highly nonpermissive to adenovirus infection. In separate experiments, we found that we were not able to infect the nonresponding K562 cells significantly with recombinant A/C/β-Gal adenovirus, while other cell lines, including the control Hep G2 line and those showing a strong p53 effect, were readily infectable (Harris *et al.*, in preparation). Thus, at least part of the variability of efficacy would appear to be due to variability of infection, although other factors may be involved as well. For example, Chen *et al.* (1991) reported that wild-type p53 can suppress tumorigenicity without inhibiting the growth rate of some tumor lines. Alternatively, mutations of regulatory proteins acting downstream from p53 may also exist in some tumor cell lines, limiting the effect of p53 treatment. The lack of a p53-specific effect in the wild-type control cell line Hep G2 is encouraging, suggesting that overexpression of wild-type p53 over endogenous background levels may have only minor effects in normal cells infected with the recombinant.

The ability to treat human cancer cells *ex vivo* and suppress their growth *in vivo* when implanted into an animal is an important step toward identifying promising gene therapy candidates. The results observed with the A/M/N/53 virus in Fig. 5 demonstrates that complete suppression is possible in an *in vivo* environment. The resumption of tumor growth in 2 out of the 4 p53-treated animals at the lower moi most likely resulted from a small percentage of cells not initially infected with the p53 recombinant at this dose. We did not analyze the resulting tumors for the presence of adenoviral genomes. The complete suppression seen with A/M/N/53 at the highr dose, however, shows that the ability of tumor growth to recover can be overcome.

p53/Adenovirus in vivo efficacy

Work presented here and by other groups (Chen *et al.*, 1990; Takahashi *et al.*, 1992) have shown that human tumor cells lacking expression of wild-type p53 can be treated *ex vivo* with p53 and result in suppression of tumor growth when the treated cells are transferred into an animal model. This report presents the first evidence of tumor suppressor gene therapy of an *in vivo* established tumor, resulting in both suppression of tumor growth and increased survival time. Delivery to tumor cells did not rely on direct injection into the tumor mass. Rather, p53 recombinant adenovirus was injected into the peritumoral space, and p53 mRNA expression was detected within the tumor. p53 expressed by the recombinants was functional and strongly suppressed tumor growth as compared to that of control, non-p53-expressing adenovirus-treated tumors. However, both p53 and control virus-treated tumor groups showed tumor suppression as compared to buffer-treated controls. It has been demonstrated that local expression of tumor necrosis factor (TNF), interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, or IL-7 can lead to T-cell-independent transient tumor suppression in nude mice (Hoch *et al.*, 1992). Exposure of monocytes to adenovirus results in the release of TNF, and adenovirus virions are also weak inducers of IFN-α/β (for review, see Gooding and Wold, 1990). Therefore, it is not surprising that we observed some tumor suppression in nude mice even with the control adenovirus. We did not observe this virus-mediated tumor suppression in the *ex vivo* control virus-treated Saos-2 tumor cells described earlier. The p53-specific *in vivo* tumor suppression was dramatically demonstrated by continued monitoring of the animals in Fig. 7. The survival time of the p53-treated mice was significantly increased, with 5 out of 5 animals still alive more than 135 days after tumor cell inoculation compared to 0 out of 5 adenovirus control-treated animals. Two out of 5 mice continue to survive beyond day 170, more than twice the survival time of the longest-lived control virus and buffer-treated animals. The surviving animals still exhibit growing tumors, which may reflect cells not initially infected with the p53 recombinant adenovirus. Higher or more frequent dosing schedules may address this. In addition, promoter shutoff (Palmer *et al.*, 1991) or additional mutations may have rendered these cells resistant to the p53 recombinant adenovirus treatment.

Implications for gene therapy

There will be over one million new cases of cancer diagnosed this year, and half that number of cancer-related deaths (American Cancer Society, 1993). p53 mutations are the most common genetic alteration asociated with human cancers, occurring in 50–60% of human cancers (Bartek *et al.*, 1991; Hollstein *et al.*, 1991; Levine, 1993). The goal of gene therapy in treating p53-deficient tumors is to reinstate a normal, functional copy of the wild-type p53 gene so that control of cellular proliferation is restored. p53 plays a central role in cell cycle progression, arresting growth so that repair or apoptosis can occur in response to DNA damage. The possibility of using p53/adenovirus to drive tumor cells into the apoptotic pathway is intriguing. Wild-type p53 has recently been identified as a necessary component for apoptosis induced by irradiation or treatment with some chemotherapeutic agents (Lowe *et al.*, 1993a,b). Due to the high prevalence of p53 mutations in human tumors, it is possible that tumors which have become refractory to chemotherapy and irradiation treatments may have become so due in part to the lack of wild-type p53. By resupplying functional p53 to these tumors, it is possible that they will now become susceptible to apoptosis normally associated with the DNA damage induced by radiation and chemotherapy.

One of the critical points in successful human tumor suppressor gene therapy is the ability to affect a significant fraction of the cancer cells. Toward that goal, recombinant adenoviruses have distinct advantages over other gene delivery methods (for review, see Siegfried, 1993). Adenoviruses have never been shown to induce tumors in humans and have been safely used as live vaccines (Straus, 1984). Replication-deficient recombinant adenoviruses can be produced by replacing the E1 region necessary for replication with the target gene. Adenovirus does not integrate into the human genome as a normal consequence of infection, thereby greatly reducing the risk of insertional mutagenesis possible with retrovirus or AAV vectors. This lack of stable integration also leads to an additional safety feature in that the transferred gene effect will be transient, as the extrachromasomal DNA will be gradually lost with continued division of normal cells. Stable, high-titer recombinant adenovirus can be produced at levels not achievable with retrovirus or AAV, allowing enough material to be produced to treat a large patient population. Others have shown that adenovirus-mediated gene delivery has a strong potential for gene therapy for diseases such as cystic fibrosis (Rosenfeld *et al.*, 1992; Rich *et al.*, 1993) and α_1-antitrypsin deficiency (Lemarchand *et al.*, 1992). Although other alternatives for gene delivery, such as cationic liposome–DNA complexes, are also currently being explored, none as yet appear as effective as adenovirus-mediated gene delivery.

Here, we have shown that recombinant adenoviruses expressing wild-type p53 can efficiently inhibit DNA synthesis and suppress the growth of a broad range of human tumor cell types, including clinically relevant targets. Furthermore, we have shown that the recombinant adenoviruses can express p53 in an *in vivo* established tumor without relying on direct injection into the tumor or prior *ex vivo* treatment of the cancer cells. The p53 expressed is functional and effectively suppressed tumor growth *in vivo* and significantly increased survival time in a nude mouse model of human lung cancer. Although further studies are needed to ensure the safety of this method of gene delivery and address possible problems of immune responses, the data presented here strongly support the concept of adenovirus-mediated p53 gene therapy of p53-deficient tumors in humans.

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Impact of human neutralizing antibodies on anti-tumor efficacy of an oncolytic adenovirus in a murine model

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ABSTRACT

Purpose: To assess the impact of anti-adenovirus neutralizing antibodies (AdNAbs) on the distribution, tolerability, and efficacy of intravenously administered of oncolytic adenovirus. A translational model was developed to evaluate the impact of humoral immunity on intravenous (IV) administration of oncolytic adenovirus in humans.

Experimental design: Initially, SCID/beige mice were passive immunized with various amount of human sera to establish a condition of pre-existing humoral immunity similar to humans. A replication deficient adenovirus encoding beta-galatosidase (rAd-βgal) was injected by IV route into these mice. An AdNAb titer that mitigated galatosidase transgene expression was determined. A xenograft tumor bearing nude mouse model was developed to assess how a similar in vivo titer would impact the activity of 01/PEME, an oncolytic adenovirus virus, following IV administration.

Results: In SCID/beige mice, there was a dose-dependence between AdNAbs and galatosidase transgene expression; 90% of transgene expression was inhibited when the titer was 80. A similar titer reconstituted in the nude mice with human serum, as was done in the SCID/beige mice, did not abrogate the anti-tumor efficacy of the replicating adenovirus following IV administration. Viral DNA increased in tumors over time.

Conclusions: In IV administration, pre-existing rAdNAb titer of 80 significantly attenuated the activity of a 2.5 $x10^{12}$ particles/kg dose of non-replicating adenovirus; the same titer had no affect on the activity of an equivalent dose of replicating adenovirus. Our results suggest that a majority of patients with pre-existing adenovirus immunity would be candidates for intravenous administration of oncolytic adenovirus.

INTRODUCTION

Oncolytic replicating adenoviruses are emerging as a promising form of anti-cancer therapy. Oncolytic adenoviruses act by replicating within and lysing tumor cells. Intratumoral replication increases the local concentration of the virus at the tumor site (1, 2). In order to treat disseminated disease, it is preferable to deliver the adenovirus intravenously. However, previous reports on the effect of pre-existing humoral immunity on intravenously administered adenovirus suggest that the neutralizing effect may prevent or significantly diminish the therapeutic value (3-5). In this study, we developed a model to test whether or not pre-existing humoral immunity representative of that found in the general population would be sufficient to neutralize the antitumor efficacy of a replicating oncolytic adenovirus.

Antibodies to adenoviruses are common in the human population due to natural infection. Studies have shown that >90% of the human population is seropositive to adenoviruses of one or more serotype by ELISA or by neutralization assay (3, 6-8). However, the anti-adenovirus antibody responses in the general population are quite variable, with AdNAb titers range from undetectable to very high (3, 7, 8). Therefore, modeling the effect of pre-existing adenovirus immunity on systemic administration of therapeutic adenoviruses is challenging.

We wished to model the effect of pre-existing humoral immunity to adenovirus on anti-tumor efficacy of an oncolytic adenovirus. To this end, a human tumor xenograft nude mouse model was developed to model pre-existing humoral immunity to adenovirus observed in the human population. As a first step in model development, SCID/beige mice were passively immunized with different pools of human sera to establish a

condition of pre-existing immunity. Because SCID/beige mice lack a humoral immune response, the only source of AdNAb titer in this model was the human sera used for passive immunization. A non-replicating adenovirus vector encoding β-galactosidase (rAd-βgal) was administered intravenously to determine the relationship between circulating AdNAb titer and vector gene expression in the liver.

A human tumor xenograft nude mouse model was developed to evaluate the inhibitory effect of human AdNAbs on the anti-tumor efficacy of an oncolytic adenovirus administered by the IV route. Pre-existing immunity in this model was generated by passive immunization using the same procedure as performed in SCID/beige mice. Because nude mice are capable of mounting a humoral response in the absence of T helper cells (9-13), mouse AdNAbs are induced following virus treatment and persist at increased neutralizing titers, analogous to the response seen in patients undergoing treatment with oncolytic adenoviruses (14-16). The data generated in these studies provide a method for estimating the feasibility of treating cancer patients with humoral immunity to adenovirus and suggest that pre-existing neutralizing antibodies would not preclude IV administration of oncolytic adenovirus for greater than 70% of the population.

MATERIALS AND METHODS

Adenovirus vectors. rAd-GFP, encoding green fluorescent protein, and rAd-βgal, encoding β-galactosidase, are E1 deleted replication deficient rAd-vectors (17). The oncolytic adenovirus 01/PEME has been described previously (18). All vectors were purified by column chromatography (19) and quantified by Resource-Q HPLC (20).

Neutralizing antibody (NAb) assay. A previously described assay (6) was used to determine the titer of AdNAb in serum. The nAb titer, defined as ID_{50}, for each serum sample was reported as the reciprocal dilution that inhibited rAd-GFP mediated transduction by 50%. Titer below 20 was extrapolated from the dilution curve.

Studies with human sera. Blood samples from healthy normal blood donors were collected for the study from the San Diego Blood Bank (San Diego, California) in accordance with the San Diego Blood Bank informed consent guidelines. 122 serum samples were collected over different times of the year. Sera were assayed individually for AdNAb titer. For passive immunization experiments, sera with titers greater than 320 were pooled into what is referred to as high titer group, and sera with titers 20–320 were pooled into what is referred to as low titer group. Sera with AdNAb titers that were below the limit of quantification (BQL) were not included in the study.

Passive immunization of human sera in SCID™-BEIGE C.B-17 mice (Taconic, Germantown, NY). Various dilutions of pooled high or low AdNAb titer human sera were used for passive immunization experiments. Sera (1.9µl-150µl) were diluted to total volume of 500 µl with vPBS (phosphate-buffered saline supplemented with 3% sucrose and 2mM of $MgCl_2$) and injected by intraperitoneal route into SCID/beige mice (n=4 for each condition). Mice were bled 3 hours post passive immunization to ensure the presence of AdNAbs in the circulation. The next day, all mice received $5x10^{10}$ particles rAd-βgal by IV route. Mice were sacrificed at 72 hours. Livers were harvested and immediately frozen for PCR analysis; portions of the livers were also fixed in OCT. Cryosections of the liver from OCT blocks were incubated with

X-gal substrate to assess β-galactosidase activity as a measure of *in vivo* transduction efficiency. Naïve mice, mock passively immunized with vPBS, were used as controls.

Human Xenograft PC3 tumor model. Athymic nude mice were obtained from Harlan Laboratories, Inc. (Indianapolis, Indiana) between the ages of 6-8 weeks old. Xenograft tumors were established by subcutaneous injection of $5x10^6$ PC3 cells (human prostate adenocarcinoma, ATCC CRL-1435) in the flanks of athymic mice. Between day 7 and 10 tumors reached $100mm^3$ in size, the mice were randomized and divided into six groups of 8 mice each. 50 µl of high titer sera diluted 1:10 in vPBS and 500 µl was injected by intraperitoneal route into two groups of 8 animals each (high titer groups). The same procedure was performed with low titer sera (low titer groups). The control groups of sham passively immunized (naïve) mice received 500 µl of vPBS. Three hours following passive immunization, all animals were bled to determine the *in vivo* AdNAb titers. The mice were then treated with two different regimens of 01/PEME: Each of the high, low and control groups were treated with a single bolus dose of $5x10^{10}$ particles 01/ PEME per animal or $1x10^{10}$ particles 01/ PEME per animal per day for five consecutive days. Tumors from all groups of mice were measured every week after 01/PEME treatment. Tumor volume was calculated assuming spherical geometry. Mean tumor size for each treatment group ± standard error of the means was plotted vs. time after cell injection.

Quantitative PCR (QPCR). Real Time Quantitative PCR and RT-PCR for rAd-β-gal was described in detail previously (6), as well as the PCR for measuring adenovirus DNA with hexon primers (21). The result of QPCR is expressed as DNA copies/mg tissue.

Toxicity evaluation. PC3 tumor bearing athymic mice were monitored for general health appearance. Mice were observed daily for clinical symptoms such as anorexia and lethargy, and weighed once a week. At the appropriate sacrifice time, blood samples were collected and tested for alanine aminotransferase (ALT) levels to evaluate liver function.

Statistics. Results are expressed means +/- standard error of the means. ANOVA *post hoc* Fisher PLSD unpaired t-test (Statview, Abacus) was used to compare difference between groups. Statistical significance was taken for P values of less than 0.05.

RESULTS

Characterization of neutralizing antibody titers against adenovirus in a population of healthy blood donors. A majority of the human population has pre-existing humoral immunity to adenovirus due to natural infection. Analysis of neutralizing antibody titers in the general population may provide an initial assessment of the percentage of the population that could benefit from gene therapy using adenovirus. Recently, we and others developed titration assays that measure neutralization of the Ad5-based vector rAd-GFP as reduction of GFP fluorescence in infected cells (6, 22). Results of these studies are in general agreement with studies using the plaque reduction assay (5, 14, 23) or the Ad-βgal neutralization assay (7), and show that approximately two-thirds of study subjects had detectable neutralizing antibodies against Ad5. In addition, we and others showed that in human sera the reduction of GFP fluorescence in the *in vitro* assay was dependent on antibodies that recognized adenovirus capsid proteins (6, 16, 24). In the present study, we determined the mid-point titers (ID_{50}) of neutralizing

antibodies from 122 healthy blood donors using serial dilutions of sera in the rAd-GFP assay. Consistent with previous studies, we found that the neutralizing antibody (NAb) titers (ID_{50}) from our study population could be divided into three groups. Based on our criteria, 48 donors had titers that were below the limit of quantification (BLQ), 45 donors had low AdNAb titer with an ID_{50} range of 20–320, and 29 had high AdNAb titer with ID_{50} >320, Fig. 1a.

AdNAb titer measured in sera of SCID/beige mice after passive immunization with human sera. To avoid any bias associated with possible idiosyncratic antibody responses, we pooled sera from four representative donors from either the high or low titer groups and ran full dilution curves using the *in vitro* rAd-GFP assay (data not shown). The resulting titers of the pooled low and high titer sera were determined to be 80 and 1560 respectively. Increasing volumes of the pooled low and high titer sera were administered intraperitoneally to SCID/beige mice to generate humoral immunity to adenovirus by passive immunization. Three hours after administration of the pooled human sera, serum samples were taken from passively immunized mice with the equivalent of 1.9 µl, 5.5µl, 16.6µl, 50µl, and 150µl of human sera. The respective titers determined in the high titer groups were as follows: below limit of quantification, <20, <20, ~89, and ~160, Fig. 1b. In mice that were passively immunized with low titer serum, only the groups that received 50µl and 150µl had quantifiable titers and both were less than 20. The ID_{50} titers determined in sera from the passively immunized mice correlated with the amount of input pooled high and low titer human sera. As expected, the circulating titers in the mice were lower due to dilution in

the mouse body fluids as well as absorption of antibodies into the tissues during homeostasis.

Effect of human neutralizing antibodies on adenovirus vector distribution following intravenous administration. In the mouse, greater than 90% of the input dose of adenovirus vector distributes to the liver following intravenous administration (25). To determine whether this distribution was affected by circulating anti-adenovirus antibodies, we challenged the passively immunized SCID/beige mice with intravenous rAd-βgal and measured the levels of rAd-βgal DNA in livers by PCR 3 days later. For the low titer groups, there was no statistically significant reduction in the amount of rAd-βgal DNA distributed to the livers with increasing doses of serum used for passive immunization (data not shown). In contrast, in groups passively immunized with high titer serum, there was a modest dose-dependent drop in viral DNA in the livers of groups passively immunized with 1.9μl, 5.5μl, and 16.6μl of serum (Fig. 2a). In mice that received 50μl of serum, viral DNA in the liver was reduced by half compared to the vehicle group. In mice that received 150μl of serum, there was a significant inhibition of viral DNA uptake in the livers of animals that received the 150μl dose of serum ($p<.05$).

Effect of human neutralizing antibodies on adenovirus vector function following intravenous administration. We assessed the impact of AdNAbs on vector function by measurement of βgal RNA in liver homogenates and by measurement of β-galactosidase activity in liver sections. Although rAd-βgal DNA was detected in the livers of all the groups, β-galactosidase activity was only detected in groups that received

less than 50μl of high titer serum. Consistent with the β-galactosidase activity, βgal RNA was significantly reduced in the group that received 50μl of high titer serum, this corresponded to a 90% inhibition of βgal activity at an in vivo titer of ≥ 80, (Fig. 2b). No reduction in βgal RNA was observed using the pooled low titer sera up to the maximum dose tested of 150 μl which had a titer of ≤20 (data not shown). Overall, there was a good correlation between circulating antibody titers measured in the *in vitro* rAd-GFP assay (Fig. 1b) and inhibition of βgal RNA expression in mouse liver following intravenous challenge with $5x10^{10}$ particles of rAd-βgal. In addition, the levels of βgal RNA in liver homogenates was in good agreement with the level of β-galactosidase expression as measured by X-gal staining of liver sections (Fig. 2c). Together these results showed that passive immunization with 50 μl of pooled human high titer sera provided levels of circulating AdNAbs sufficient to inhibit rAd-mediated gene expression in the livers by 90% and further demonstrated that titers measured using the *in vitro* rAd-GFP neutralization assay could be correlated to levels of *in vivo* transgene expression following intravenous administration of a non-replicating adenovirus vector.

Passive immunization against adenovirus in a nude mouse tumor model.

Oncolytic adenoviruses such as 01/PEME have shown significant anti-tumor activity in animal models using intravenous administration (21). Because human adenovirus does not replicate in mouse cells or murine tumors, most preclinical studies with oncolytic adenovirus have been done using human tumor xenografts in nude mice. To explore the effect of pre-existing humoral immunity on oncolytic adenovirus distribution and function in the human tumor xeonograft/nude mouse model, we passively immunized

tumor bearing nude mice as described above for the SCID/beige mouse studies. Human PC3 prostate tumor bearing mice were passively immunized by i.p. injection of pooled human sera from either the high titer or low titer groups. The 50 µl dose of human serum was selected because this provided approximately 90% reduction of virus gene expression for the high titer group. Samples of serum were taken from the passively immunized mice 3 hours post administration for determination of circulating neutralizing titers. The circulating titers for mice passively immunized with the high and low titer human sera were very comparable to those observed in the SCID/beige mice using comparable doses of sera (Fig. 3a). Mice from the high titer group had circulating AdNAb titers of 100, mice from the low titer group had circulating titers <20 and mice injected with vehicle had below quantification titer.

Effect of human neutralizing antibodies on distribution and replication of oncolytic adenovirus following intravenous administration. We evaluated the impact of circulating AdNAbs on distribution of an oncolytic adenovirus, 01/PEME (18), to the liver and tumor in the human PC3 xenograft/nude mouse model using passive immunization with pooled human sera as described above. Oncolytic adenovirus 01/PEME was then administered by tail vein injection. Doses of either $1x10^{10}$ particles or $5x10^{10}$ particles 01/PEME were administered as single bolus IV injection. The $1x10^{10}$ particle dose was selected because it was previously shown to be the efficacious dose in the PC3 tumor model in non-immunized nude mice (21). A 5-fold higher dose also was selected because of the anticipated reduction in anti-tumor efficacy due to passive immunization with human AdNAbs.

The amount of 01/PEME viral DNA in livers and tumors was measured by quantitative PCR three hours and five days post administration. These time points were selected based on previous studies showing that distribution of 01/PEME to PC3 tumors in nude mice was detectable at 3 hours and that replication of 01/PEME DNA within the tumors peaked at day 5 (21). At the three-hour time point, similar levels of viral DNA were detected in the livers of PC3 tumor bearing nude mice regardless of their circulating AdNAb titers (Fig. 3b). As expected, the level of viral DNA in the liver was lower in the $1x10^{10}$ particle dose groups compared to the $5x10^{10}$ particles dose groups. By day 5, there was a significant decrease in viral DNA in the livers of all the groups that received the $1x10^{10}$ particles dose ($p<0.05$), Fig. 3b gray columns. For the $5x10^{10}$ particle dose group, viral DNA levels on day 5 remained similar to those observed at the 3-hour time point, and persisted until day 12 (data not shown).

Viral DNA distributed to the tumor was approximately 500 times less than that observed in the liver at the 3 hour time point. Differences in administered dose of 01/PEME ($1x10^{10}$ particles vs. $5x10^{10}$ particles) resulted in corresponding differences in levels of viral DNA detected in tumors at the 3 hour time point (Fig. 3c). Circulating AdNAb titers had no statistically significant impact on the amounts of viral DNA distributed to tumors at this time point; the amount of virus delivered was not correlated with AdNAb titers. However, significant increases in viral DNA in tumors was observed at day 5 in all treatment groups ($p<0.05$), regardless of the status of pre-existing immunity or the administered dose. The group with the $1x10^{10}$ particle dose in the low titer group had a modest increase in viral DNA on day 5, $p<0.07$.

Endogenous anti-adenovirus neutralizing antibodies were induced in tumor bearing nude mice after IV administration of 01/PEME. Induction of neutralizing antibodies to oncolytic adenovirus has been described in several clinical studies (14, 15, 26). Although we were unable to model induction of *human* neutralizing antibodies in mouse models, it was possible to model some aspects of induction of neutralizing antibodies in response to therapy. Athymic nude mice are deficient in most T-cell mediated immune responses but remain capable of mounting humoral responses to antigen. To evaluate the development of an endogenous humoral response to adenovirus in mice that had pre-existing humoral immunity, we measured circulating AdNAb titers in sera of tumor bearing nude mice 5 days after IV administration of 01/PEME. As shown in Table 1, neutralizing antibody responses to adenovirus developed by day 5 in the sham passively immunized (naïve) mice injected with saline vehicle instead of human serum. The circulating neutralizing titer (due to endogenous mouse antibodies) induced in the sham-passively immunized groups exceeded the circulating neutralizing titers generated by passive immunization with human serum. The vehicle group injected with the $5x10^{10}$ particles dose induced a higher AdNAb response (ID_{50}=300) than the group injected with the $1x10^{10}$ particles dose (ID_{50}=152). Interestingly, in both $1x10^{10}$ and $5x10^{10}$ particles groups, mice that were passively immunized with the low titer human sera induced higher AdNAb response than either the high titer or the vehicle groups. We had previously determined that the half life of in vivo anti-AdNAbs in human passive immunized sera was 14 days (data not shown), thus the circulating neutralizing activity present by day 5 in the passively immunized groups was due to mouse endogenous AdNAbs plus residual human AdNAbs.

PC3 efficacy tumor model in the presence of established humoral immunity to adenovirus. In a study of pharmacological indicators of antitumor efficacy of 01/PEME, efficient distribution to the tumor site was found to be a critical factor for efficacy of oncolytic adenoviruses (21). Similar to what was seen in the SCID/beige model, circulating AdNAb titers $\leq$80 did not impact significantly 01/PEME distribution to the tumor or to the liver in the human xenograft nude model. However in the SCID/beige mice, circulating AdNAb titers $\geq$ 80 were found to inhibit adenovirus transgene expression in the liver by 90%. To determine whether AdNAbs would inhibit the antitumor efficacy, we followed tumor growth over time in passively immunized human xenograft- nude mice (as described above) treated with 01/PEME.

To examine the effect of pre-existing AdNAb titer on the outcome of 01/PEME antitumor efficacy, tumor-bearing nude mice were passively immunized with high titer and low titer human sera prior to receiving 01/PEME therapy. Two regimens of therapy were used for the anti-tumor efficacy study: A single bolus IV injection of $5x10^{10}$ particles of 01/PEME or five consecutive daily injections of $1x10^{10}$ particles of 01/PEME. As seen in Fig. 4a, by day 37, all groups receiving 01/PEME had tumor growth inhibited by 70% regardless of pre-existing AdNAb titer status (derived from passive immunization of human sera) and regardless of the induction of endogenous AdNAb by day 5 (derived from IV administration of 01/PEME).

Impact of pre-existing immunity on vector induced toxicity after IV administration of 01/PEME. Progression of PC3 tumors in this nude mouse model was associated with progressive loss of body weight and other signs of cachexia. Systemic

administration of oncolytic adenoviruses was also associated with elevation of serum levels of liver enzymes (e.g., ALT) and inflammatory cytokines, including TNF-α (25, 27-30).

The anti-tumor effect of 01/PEME was correlated with weight gain in animals treated with 01/PEME, regardless of circulating AdNAb titer (Fig. 4b). No signs of anorexia or lethargy were observed in the 01/PEME-treated animals. In contrast, untreated animals lost body weight as their tumors progressed.

We assessed the effect of circulating AdNAbs on serum liver enzyme ALT levels in mice treated with single bolus IV administration of $1x10^{10}$ or $5x10^{10}$ particles of 01/PEME. Sera were collected from 01/PEME treated mice three hours and 5 days post IV administration. In animals of all the groups that received $1x10^{10}$ particles of 01/PEME by IV treatment, the serum ALT levels were within the normal range (ALT: 20-60 U/L) measured at 3 hours and on day 5 (data not shown). At the $5x10^{10}$ particle dose of 01/PEME, ALT levels were within normal range at 3 hours (ALT: 20-50U/L), but rose significantly by day 5 in all the 01/PEME treated groups (ALT: 199-530U/L), $p<0.05$ (Fig. 5). The circulating AdNAbs in the passively immunized mice had no significant effect on ALT response when compared to sham immunized mice at either the $1x10^{10}$ or $5x10^{10}$ particle 01/PEME dose.

Discussion

Preclinical models used to study oncolytic adenoviruses such as 01/PEME for cancer therapy test for the ability of the adenovirus to be delivered to the tumor site, replicate in the tumor, and inhibit tumor growth in a dose dependent manner. Because

replication of oncolytic adenoviruses is restricted to human cells, preclinical testing is generally performed using human tumor xenografts in immune-compromised mice. These approaches have yielded useful information on the relative safety and efficacy of systemic delivery of oncolytic adenovirus. However, the immune-compromised mouse models omit many aspects of the host immune response that may be relevant to the distribution, tolerability, and efficacy of oncolytic adenovirus.

In contrast to the very potent neutralizing humoral response to adenovirus seen in immune-competent mice, we found that a majority of humans exposed to adenovirus through natural infection did not have high levels of AdNAb present in the serum (3, 6, 7). The model used in this study was designed to address the impact of naturally-acquired pre-existing immunity in cancer patients undergoing adenovirus gene therapy. In the human tumor xenograft nude mice model, we could assess the delivery and the replication of 01/PEME in the presence of pre-existing AdNAb (from passive immunization of human serum) and in the presence of newly induced mouse AdNAb from the host (in response to IV administration of 01/PEME), conditions analogous to those experienced by cancer patients undergoing virus therapy. From this model, we were able to follow the distribution, the tolerability, and the efficacy of 01/PEME post IV administration in the presence of humoral immune responses.

The use of human sera in these translational models addressed how the different AdNAb titers found in the human population would affect systemic oncolytic adenovirus therapy. Our data showed that the presence of anti-adenovirus NAbs did not contribute or prevent hepatic toxicity as reported in studies using pre-immunized animal models (5, 31). Rather, our data was very similar to several human clinical trails involving different

replicating competent adenoviruses delivered by various intra-vascular routes (30). The increase toxicity such as transaminitis was associated with higher dose of virus used (as in our $5x10^{10}$ particles dose) and not associated with the presence of AdNAbs found in the host.

Our data was consistent with previous reports from clinical trials with systemic administration of oncolytic adenoviruses. AdNAB titers increased following IV administration regardless of the pre-existing humoral immunity (14-16, 30, 32). However, the in vivo titrations of AdNAbs in our SCID/beige study allowed evaluation of the effect of pre-existing immunity on distribution of the virus to the liver. Gene expression in the liver was inversely proportional to the *in vivo* AdNAb titers, and 90% of the viral function was inhibited when the *in vivo* titer was >80. This indicated that AdNAbs have a significant effect in virus function after IV administration, and that measuring virus particles in the blood or target organs during viral therapy by quantitative PCR alone would not give sufficient assessment of the bioactivity of the virus. For oncolytic adenovirus therapy, functional assays that can quantify infectious viral particles in the presence of autologous patients' sera may help to elucidate some aspects of viral drug activities

In the nude mouse/xenograft model, anti-tumor efficacy was achieved in the presence of a blocking AdNAb titer. The replication of 01/PEME in the tumors is similar in the naïve as in the low titer and high titer group (Fig. 3b). These results suggest that AdNAbs may not play as critical a role in inhibiting the activities of the 01/PEME within the tumor microenvironment as they are in the periphery. It is likely that without the replication ability of the adenovirus, AdNAbs would attenuate the small number of viral

particles distributed to the tumors after IV delivery. Alternatively, some aspect of the host innate immune responses may contribute to anti-tumor efficacy (33-35). Further studies are needed to elucidate the host immune mechanisms involved in the anti-tumor efficacy of 01/PEME.

There are limitations in our models. Immune responses induced by oncolytic adenovirus in systemic administration would best be done in immune competent models as demonstrated recently by Hallden et al (34). However, it would be difficult to replicate the condition of natural infection by human adenovirus in laboratory animals. Our models are also limited in assessing induced humoral immunity resulting from systemic administration of oncolytic adenovirus because the induced antibodies are of mouse origin. Although induction of mouse AdNAbs recapitulates the increase in neutralizing titer seen in patients treated with oncolytic adenoviruses, the antigen specificity, avidity and isotype preference of the induced mouse antibodies may differ from those induced in humans.

Our models highlighted some of the hurdles of systemic delivery of 01/PEME in assessing the distribution, tolerability, and anti-tumor efficacy. Anti-tumor response with 01/PEME is a balance between dose dependent toxicity and dose dependent efficacy. We have shown that 01/PEME is at least a 1000-fold more potent that a non-replicating rAd-vector such as rAd-p53 in the same tumor model (21), it is possible to achieve efficacy with a lower dose of 01/PEME, and therefore avoid hepatic toxicity. However, a lower dose of 01/PEME may not be efficacious in the presence of AdNAbs. Reduction of viral-specific AdNAbs either by direct inhibition of AdNAbs formation or as proposed by our

previous study by lowering the titer of AdNAbs by immuno-plasmapheresis would preserve virus activities (6, 36, 37).

Our models were able to provide some insights into the relationship between AdNAb titer and drug activity that have been difficult to obtain from clinical data, possibly because of variability in pre-existing immunity between patients. Our models demonstrated a correlation between circulating AdNAb titer and viral (drug) activity for replication defective vectors, but also showed that the AdNAb threshold for inhibition of oncolytic adenovirus activity is increased, most likely due to the virus to replicate at the tumor site; delivery, replication and anti-tumor efficacy of 01/PEME was maintained in the presence of AdNAb titers capable of blocking a rAd-βgal gene expression in the SCID/beige model. Because the AdNAb titers in the passively immunized mice at the time of IV administration of 01/PEME were higher than those found in greater then 70% of healthy human donors, these results suggest that pre-existing humoral immunity will not preclude systemic administration of 01/PEME in the majority of cancer patients. Importantly, our study suggests that the newly induced antibodies in patients during viral therapy should be lower than a titer of 80 before the next round of therapy. Given the safety profile accumulated so far with oncolytic adenovirus in intra-vascular delivery, modulating some aspects of the host immune responses during therapy should be tested for this type of drug to be used for systemic anti-cancer therapy.

Figure Legends

Figure 1. Characterization of neutralizing antibody titers against adenovirus in human sera and in SCID/beige mice sera passively immunized with human sera.

A. The neutralizing activities in sera of normal individuals were defined by ID_{50} titers using an *in vitro* rAd-GFP base assay. Sera from 122 donors were assayed and three patterns of neutralizing titers emerged: 48 donors had titers below the limit of quantification, 45 had low titer (ID_{50}< 320), and 29 had high titers (ID_{50}>320).

B. Passive immunization using pooled high titer and pooled low titer human sera in SCID/beige mice. ID_{50} titer values of sera taken from low titer (gray bars) and high titer (black bars) groups of SCID/beige mice that were passively immunized with different volumes of pooled low titer or high titer human sera.

Figure 2. Effect of neutralizing antibodies on adenovirus vector distribution and transgene expression following intravenous administration of $5x10^{10}$ particles of rAd-βgal. Three days post IV administration of rAd-βgal in SCID/beige mice passively immunized with different volumes of pooled high human sera, livers were harvested and rAd-βgal DNA was assessed by PCR (A) and rAd-βgal RNA was assessed by RT-PCR (B). Percent of vehicle control is calculated base on result from the sham passively immunized group. Assessment of rAd-βgal activity was done by X-gal staining of liver sections (C).

Figure 3. Effects of neutralizing antibodies on oncolytic adenovirus (01/PEME) distribution and function in tumor bearing nude mice post IV administration. PC3 tumors bearing nude mice were passive immunized with 50 μl of pooled low titer or high titer human serum 24 hours before IV treatment with of 01/PEME. ID_{50} values of sera taken

from low titer (white bars) and high titer (cross bars) groups of tumor bearing nude mice passively transferred with 50 μl of pooled low titer or high titer human sera (A). Viral distribution and function measured in liver (B) and tumor (C). Doses of vector used for IV injection: $1x10^{10}$ particles 01/PEME (gray bars) and $5x10^{10}$ particles 01/PEME (black bars). Naïve group was not treated with vector. The vehicle group had sham passive immunization and was treated with 01/PEME. * $p<0.05$ between 3 hours and day 5. Results shown are from 1 of 2 replicate experiments.

Figure 4. Inhibition of tumor-growth following IV administration of 01/PEME in PC3 tumor bearing nude mice.

A. Tumor volume measured on day 37 in animals passive immunize with high titer serum treated with a single bolus IV administration of 5 $x10^{10}$ particles of 01/PEME (open triangles), 5 daily injections of $1x10^{10}$ particles 01/PEME (closed triangles), and 5 $x10^{10}$ particles 01/PEME in sham vehicle immunized animals (open squares). Tumor volume measured on day 37 in animals with passive immunization of low titer serum followed with a single bolus IV administration of 5 $x10^{10}$ particles 01/PEME (open circles), 5 daily injections of $1x10^{10}$ particles 01/PEME (closed circles), and 5 daily injections of 1 $x10^{10}$ particles 01/PEME in sham passively immunized animals (closed squares). Untreated animals had significant increase of tumor growth (asterisk stars).

B. Weekly weight determined in animal passively immunized with high titer serum treated with a single bolus IV administration of $5x10^{10}$ particles 01/PEME (open triangles), 5 daily injections of $1x10^{10}$ particles 01/PEME (closed triangles), and $5x10^{10}$ particles 01/PEME in sham passively immunized animals (open squares).

Weekly weight determined in animals in groups passively immunized with low titer serum follow with a single bolus IV administration of $5x10^{10}$ particles 01/PEME (open circles), 5 daily injections of $1x10^{10}$ particles 01/PEME (closed circles), and 5 daily injections of $1x10^{10}$ particles 01/PEME in sham passively immunized animals (closed squares). Untreated animals had significant decrease of body weights (asterisk stars).

Figure 5. Liver alanine aminotransferase (ALT) measured in sera of 01/PEME treated PC3 tumor bearing nude mice passively immunized with high titer human sera at 3 hours (gray bars) and on day 5 (black bars) after $5x10^{10}$ particles 01/PEME by IV administration. *$p<0.05$ between 3 hours and day 5.

Table 1. Induction of anti-adenovirus humoral immune responses in PC3 tumor bearing nude mice. Vehicle groups were sham passive immunized with vPBS and thus served as mice with no pre-existing anti-adenovirus immunity. Naïve mice were not treated with 01/PEME. BLQ was below the limit of quantification.

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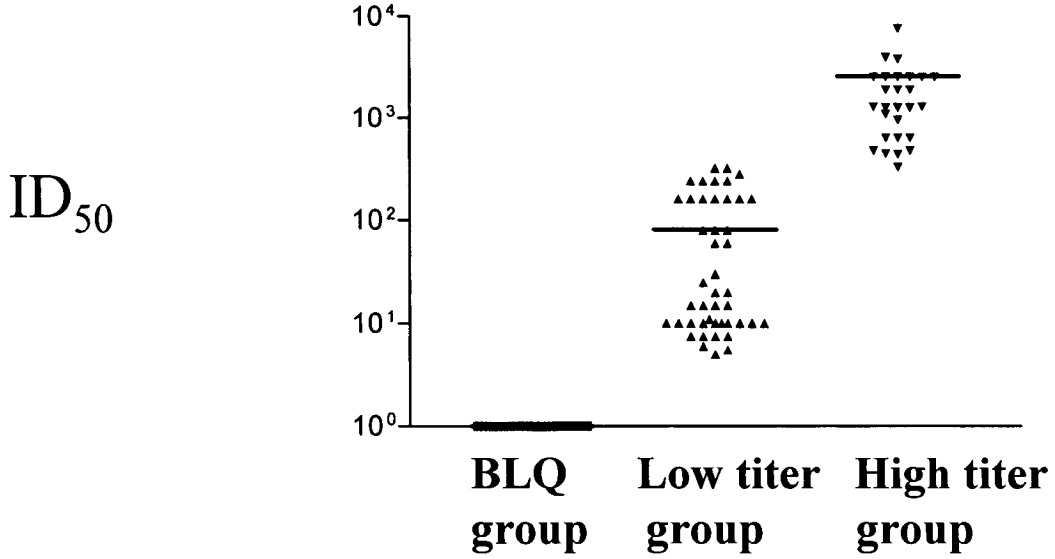

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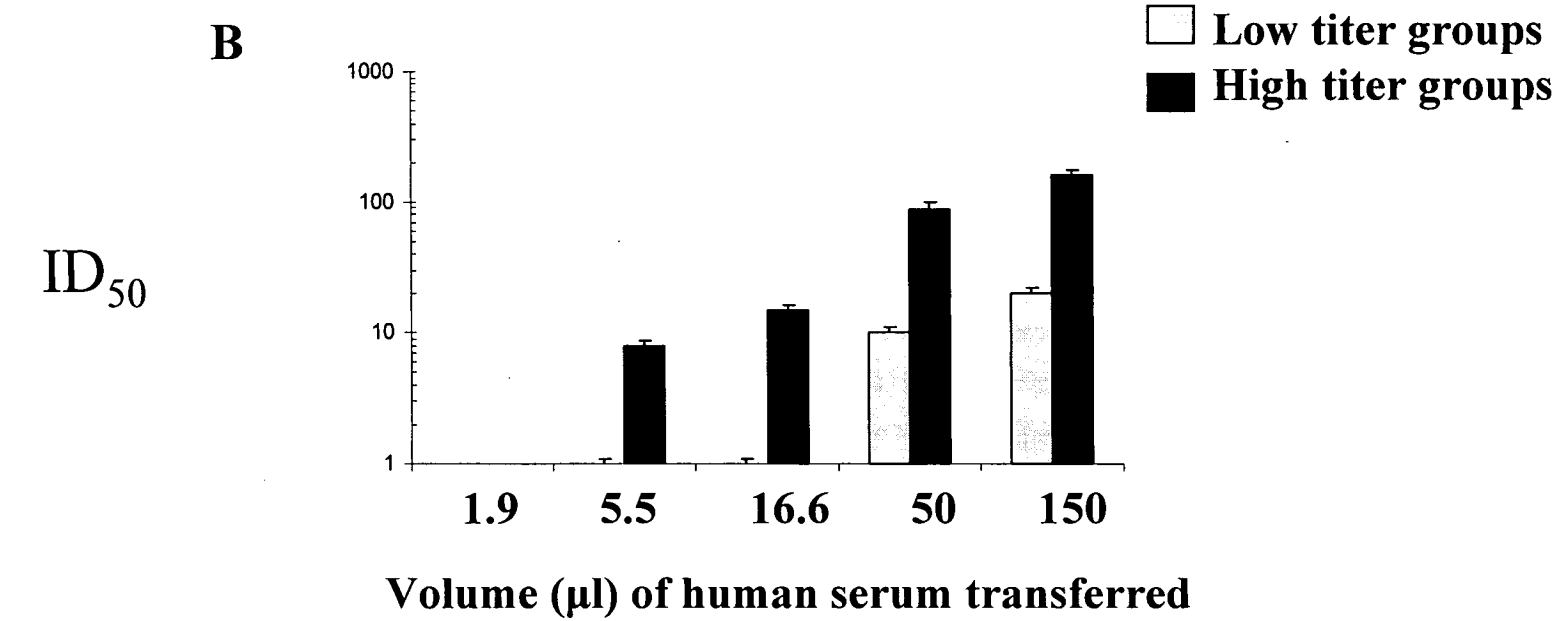

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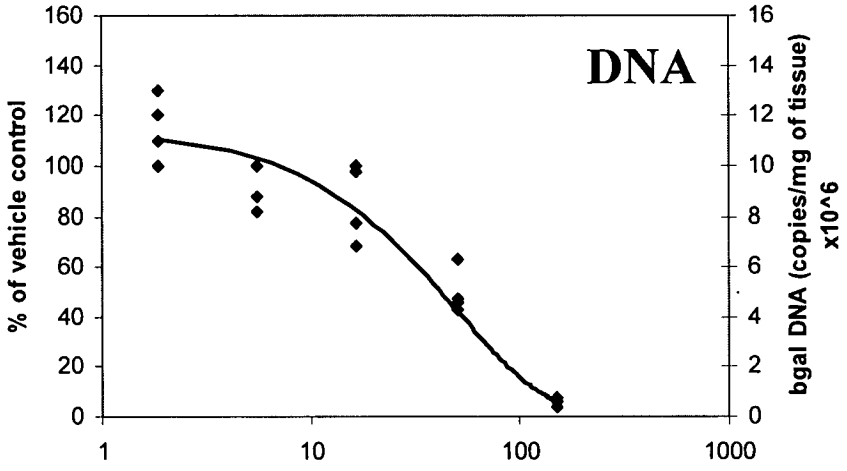

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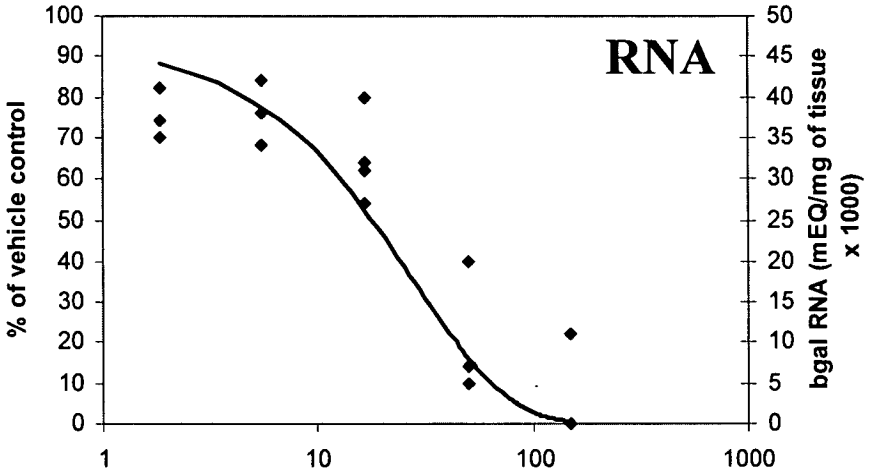

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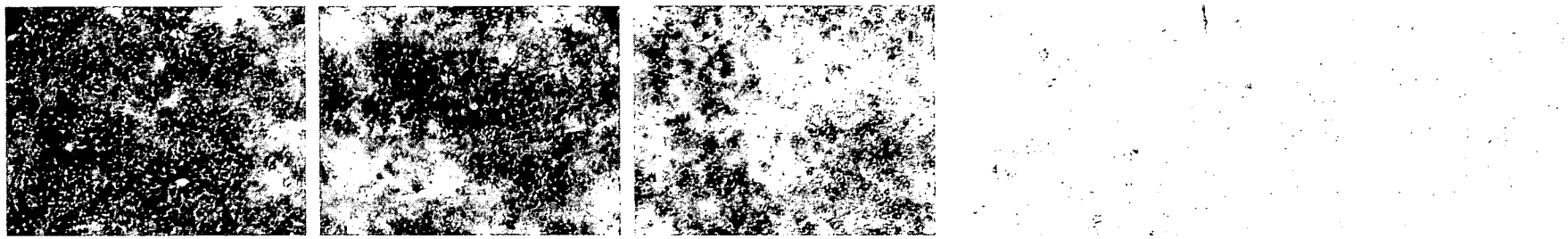

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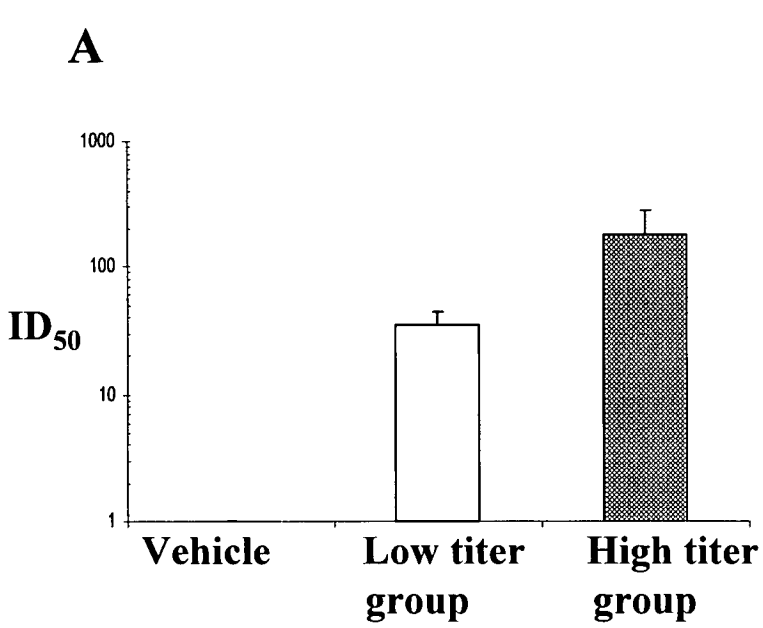

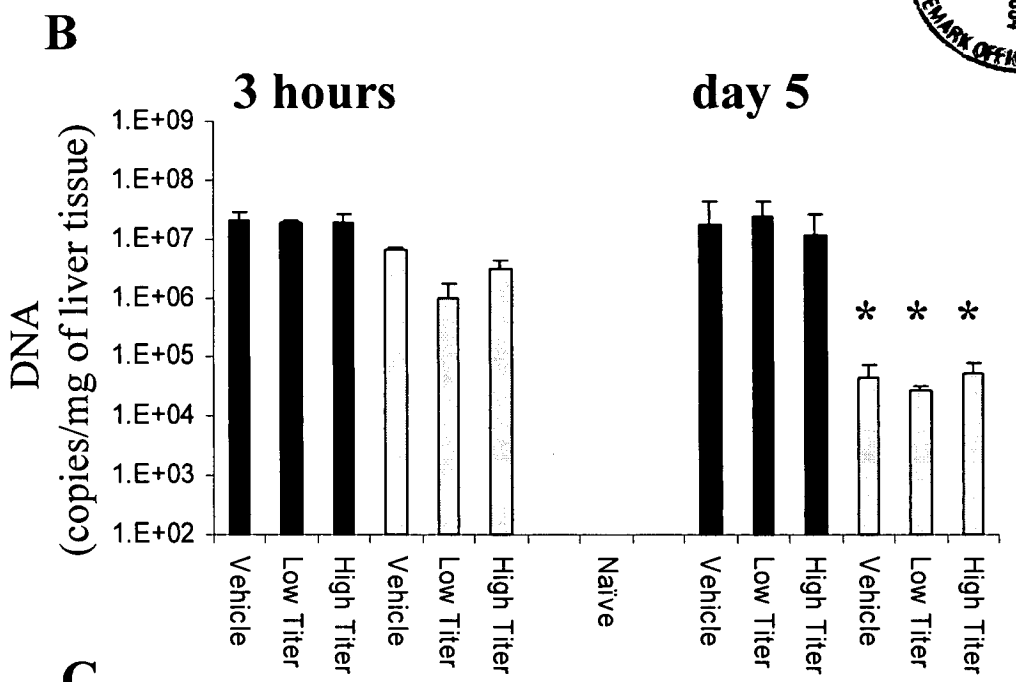

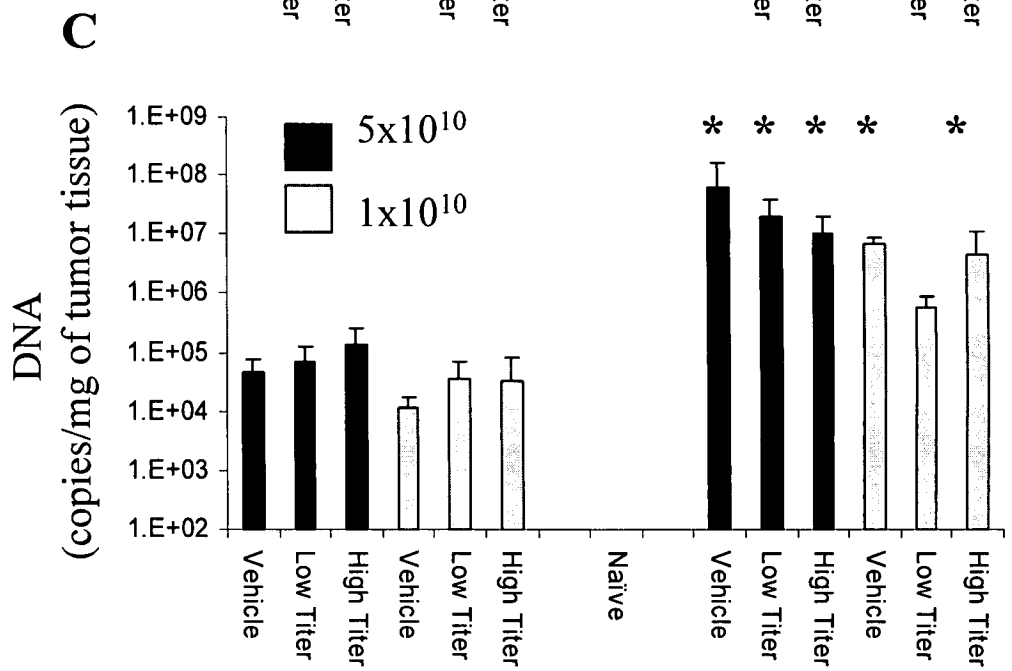

Treatment (particles/mouse)	Group	Pre-treatment (ID_{50} [a])	Post-treatment (ID_{50} [b])
$1x10^{10}$	Vehicle	BLQ	152±25
$1x10^{10}$	Low Titer	<20	520±6
$1x10^{10}$	High Titer	100	166±17
None	Naïve	BLQ	BLQ
$5x10^{10}$	Vehicle	BLQ	300±13
$5x10^{10}$	Low Titer	<20	640±27
$5x10^{10}$	High Titer	100	320±10

a. Titers measured 3 hours after passive immunization.
b. Titers measured 5 days after 01/PEME IV administration.

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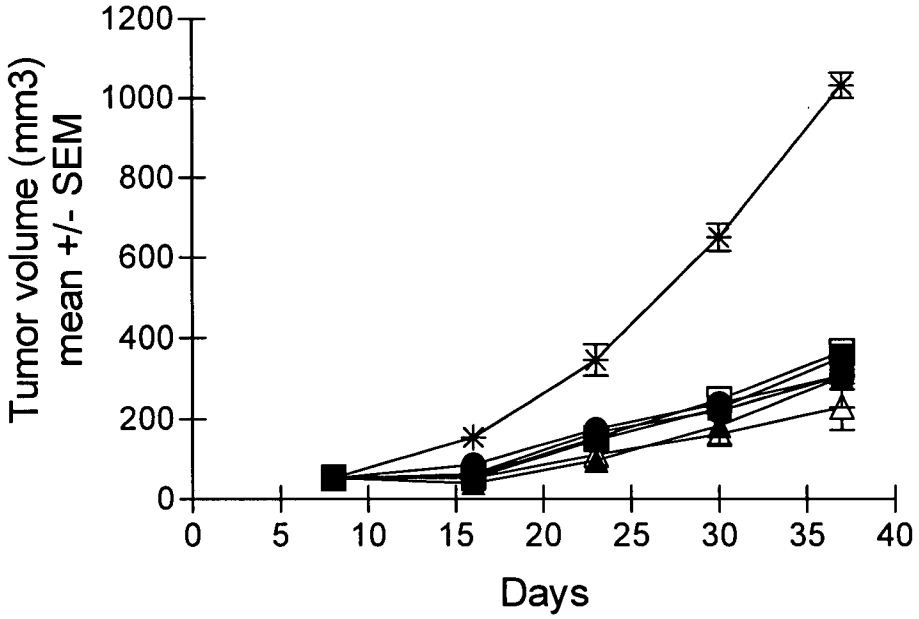

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